Reconstitution of pollinator-mediated speciation in *Petunia* through single gene mutations

Inaugural dissertation
of the Faculty of Science,
University of Bern

presented by
Martina Lüthi
from Lauperswil, BE

Supervisor of the doctoral thesis
Prof. Dr. Cris Kuhlemeier
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source: https://doi.org/10.48549/3898 | downloaded: 12.10.2024

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Accepted by the Faculty of Science.

Bern, January 27, 2022

The Dean
Prof. Dr. Zoltan Balogh

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Foreword

The experimental work presented in chapters 2 - 4 was performed during my PhD thesis at the University of Bern, Institute of Plant Sciences. The majority of the findings in these chapters are the results of my own work, however, some experimental work and data analysis has been contributed by colleagues. The individual contributions of each person are described in the author contributions section at the beginning of each experimental chapter.

Acknowledgements

Research is never a sole undertaking but a team effort and I would therefore like to thank the many people who have contributed to this work, whether it be in terms of scientific input, lab work, plant maintenance, bioinformatics support or through helpful conversations inside and outside the lab.

First of all I would like to thank my supervisor Prof. Cris Kuhlemeier for his help and support throughout this project. I am extremely grateful for having been given the opportunity to work on such an exciting project, combining molecular work with insect behavioral assays to get the best of both worlds. Thanks Cris, for allowing me the freedom to develop my own research questions and projects as well as your continuous advice during my entire PhD. My thanks also goes to Prof. Beverley Glover and Prof. Katie Peichel for taking the time to be my reviewers and serve on my thesis committee.

I would also like to express my thanks to the current and past members of the "Petunia" group: Andrea, Chaobin, Diane, Fabrizio, Gina, Jens, Korinna, Lea, Louis, Ludivine, Marta, Mathieu, Mathilde, Therese, Tracey, Viviane and Vivien. Working in a collaborative team with people of various backgrounds has helped me acquire and learn new techniques and skills and I have enjoyed working with all of you. A special thanks goes to Andrea, for your constant encouragement and help during my entire PhD; whether it be while setting up the insect facility, being my field work buddy to Brazil or helping me throughout the writing process. I could not have finished this without you! I would also like to express my thanks to the research gardeners, Christopher, Christina, Jasmin and Sarah, for taking such good care of my plants and always lending a helping hand.

Thank you as well to Prof. Loreta Brandão de Freitas and her team for welcoming us in Brazil for field work and being our Brazilian tour guides. It was impressive getting to see the Petunia plants in their natural habitats. My thanks also goes to André Arand from the University of Kassel, for showing me his hawkmoth facility and rearing setup and providing me with such excellent hawkmoth pupae for my experiments. Finally I thank Stephanie and Flurin for their help with photography questions and designing figures - you two are absolute pros with cameras.

Summary

Identifying the major genes involved in speciation processes is a key step towards understanding the molecular basis of evolution. A major component of speciation processes is reproductive isolation, which can occur in plants through differential visitation of pollinators due to diverging pollination syndromes. Pollination syndromes are defined as a suite of floral traits associated with the attraction of specific pollinators. In the genus *Petunia* multiple shifts in pollination syndromes can be observed, allowing the genetic changes underlying evolutionary shifts in pollinator attraction to be studied.

Two major clades have been defined in the *Petunia* genus: the short tube and long tube clade. The short tube clade represents the ancestral traits and encompasses the majority of the species, all with very similar phenotypes to attract bee pollinators: purple, UV reflecting flowers with a short tube, producing low amounts of scent. Species in the long tube clade exhibit different pollination syndromes and include *P. axillaris*, displaying white colored, UV absorbing flowers, emitting ample amounts of scent to attract hawkmoths, as well as *P. secreta* with purple colored, UV reflective flowers, emitting no scent and visited by bee pollinators. Transitions between these pollination syndromes in the long tube clade have been attributed to changes in few major effect genes. Specifically changes in the R2R3-MYB transcription factors *AN2* and *MYB-FL,* major determinants of visible and UV color respectively, are responsible for gains and losses of visible and UV color. UV (flavonol) and visible (anthocyanin) color pigments are produced through different branches of the same flavonoid biosynthetic pathway. The transcription factor *ODO1* and structural gene *CNL1* determine changes in scent volatiles between the species.

Here I aimed to mimic changes in phenotypic traits occurring during the transition from hawkmoth to bee pollination in the long tube clade in *Petunia*. Mimicking this transition required restoration of the *AN2* gene while rendering *MYB-FL*, *ODO1* and *CNL1* non-functional. Through multiple transgenic complementation approaches *AN2* function was restored, although full visible color as observed *in P. secreta* was not achieved. Expressing the *AN2* gene in a colorless background with the *CHS-A* promoter, a strong petal-specific promoter, induced the strongest change in visible color with expression levels of *AN2* exceeding those found in *P. secreta*. This led to elevated anthocyanidin absorbance in the

transgenic plants compared to *P. axillaris*. However, these levels were still lower than those found in the purple colored *P. secreta*. Introducing a functional *AN2* gene in a *P. axillaris* background also led to expression differences in structural genes of the flavonoid biosynthetic pathway. Genes responsible for flavonol production were downregulated while genes responsible for anthocyanin production were upregulated. However, the expression pattern in the transgenic lines was not equivalent to the one observed in *P. secreta*, underlining the need for further genes being required to restore complete visible color in a *P. axillaris* background.

Genetic modification of the *MYB-FL* gene in a *P. axillaris* background through a CRISPR/Cas9 approach rendered it non-functional, altering the floral UV phenotype from absorbent to reflective. Not only did I observe changes in UV color, but visible color changed from white to light pink as well, suggesting a trade-off between the anthocyanin and flavonol branches in the flavonoid biosynthetic pathway. I did not observe any other phenotypic effects on parameters associated with pollinator attraction, demonstrating a specific function of *MYB-FL*. Knocking-out *MYB-FL* had specific effects on gene expression of the flavonoid biosynthetic pathway: expression of target flavonol-related biosynthetic genes was reduced while expression of anthocyanin-related genes was elevated. The low pleiotropy observed when mutating *MYB-FL* was due to spatial specificity of *MYB-FL* expression, as it is highly expressed in floral limbs, but expressed at very low levels in other floral organs. Pollinator assays with primary pollinators (hawkmoths) demonstrated a significant preference towards the *P. axillaris* wildtype compared to the *myb-fl* mutant lines. The opposite preference was observed for secondary pollinators (bumblebees, solitary bees), who preferred the *myb-fl* mutant over *P. axillaris* in most cases. However, when presented with the choice of *P. secreta* vs. the *myb-fl* mutant, these two lines were not yet indistinguishable for pollinators, demonstrating the need for further phenotypic changes needed to obtain a complete *P. secreta* mimic.

To account for these further phenotypic changes, I also aimed to combine the mutated *MYB-FL* gene with mutated scent speciation genes *ODO1* and *CNL1* in a *P. axillaris* background. Knocking-out the *ODO1* transcription factor eliminated production of major scent compounds methylbenzoate, benzaldehyde and isoeugenol/eugenol, creating a scentless mutant. Mutation of the *CNL1* gene eliminated scent volatiles methylbenzoate

and benzaldehyde but did not alter production of isoeugenol/eugenol compounds. No other phenotypic effects on traits affecting pollinator visitations were observed. I developed a crossing scheme to combine these altered color and scent speciation genes to create a *P. secreta* mimic. Ultimately analyzing the *P. secreta* mimic in pollinator assays should demonstrate whether altering these few speciation genes is enough to obtain a shift in primary and secondary pollinator preference strong enough to cause reproductive isolation. This should help settle the debate about the contributions of few mutations of large effect during the process of speciation.

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Abbreviations

° C	degrees Celsius
AA	amino acid
bHLH	basic helix-loop-helix transcription factor
bp	basepairs
C	carbon molecule
CaMV35s	Cauliflower mosaic virus 35S promoter
Chr	chromosome
cm	centimeter
CTAB	cetyltrimethyl ammonium bromide
C-terminus	carboxyl terminus
d	day
DE	differentially expressed
DHK	dihydrokaempferol
DHM	dihydromyricetin
DHQ	dihydroquercetin
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide-triphosphate
g	gram
GV3101	*Agrobacterium tumefaciens* strain
h	hour
HPLC	high performance liquid chromatography
ISO	sensitivity of camera
LB	lysogeny broth
LBA4404	*Agrobacterium tumefaciens* strain
LC-UV	liquid chromatography equipped with UV diode array detector
M	molar, moles per liter
mg	milligram
min	minutes
Mitchell	*Petunia hybrida* cultivar Mitchell
ml	milliliter
mm	millimeter
mM	millimolar
MS	Murashige-Skoog medium
MYB	MYB transcription factor
µg	microgram
µl	microliter
µM	micromolar
nm	nanometer
nM	nanomolar
PCR	polymerase chain reaction
ppb	parts per billion
PTR-MS	proton transfer reaction mass spectrometry
QTL	quantitative trait locus
R2R3-MYB	R2R3-MYB transcription factor
RNA	ribonucleic acid

RNAseq	RNA sequencing
RT-qPCR	reverse transcription quantitative PCR
s	second
SDS	sodium dodecyl sulphate
SG7	subgroup 7
U6	tomato U6 promoter; RNA polymerase III promoter
U6-26	*Arabidopsis thaliana* U6-26 promoter; RNA polymerase III promoter
UV	ultraviolet
VIGS	virus-induced gene silencing
VIS	visible
WD40	WD-repeat protein
YEB	yeast extract beef

Genes, loci and enzymes

Genes are depicted in italicized font in the text, their respective enzymes in roman font. Non-functional/mutant genotypes are indicated in lowercase letters (e.g. *myb-fl*) while their wildtype counterparts are shown in uppercase letters (e.g. *MYB-FL*).

3GT	anthocyanidin 3 glucosyltransferase
4CL-A	4-COUMAROYL:COA-LIGASE-A; encodes 4-coumaroyl:CoA-ligase
4CL-B	4-COUMAROYL:COA-LIGASE-B; encodes 4-coumaroyl:CoA-ligase
4CL-C	4-COUMAROYL:COA-LIGASE-C; encodes 4-coumaroyl:CoA-ligase
4CL-D	4-COUMAROYL:COA-LIGASE-D; encodes 4-coumaroyl:CoA-ligase
5GT	anthocyanidin 5 glucosyltransferase
AAT	anthocyanidin acyltransferase
ACTIN11	Actin-11
AN1	ANTHOCYANIN1; encodes a bHLH transcription factor
AN11	ANTHOCYANIN11; encodes a WD40 repeat protein
AN2	ANTHOCYANIN2; encodes an R2R3-MYB transcription factor
AN4	ANTHOCYANIN4; encodes an R2R3-MYB transcription factor
AN9	glutathione S-transferase
ANS	anthocyanidin synthase
ART	anthocyanidin rhamnosyltransferase
BPBT	Benzoyl-CoA:benzylalcohol/2-phenylethanol benzoyltransferase
BSMT	Benzoic acid/salicylic acid carboxyl methyltransferase
C4H-A	CINNAMATE 4-HYDROXYLASE-A; encodes cinnamate 4-hydroxylase
C4H-B	CINNAMATE 4-HYDROXYLASE-B; encodes cinnamate 4-hydroxylase
CcoAOMT1	caffeoyl-CoA O-methyltransferase 1
CcoAOMT2	caffeoyl-CoA O-methyltransferase 2
CcoAOMT3	caffeoyl-CoA O-methyltransferase 3
CcoAOMT4	caffeoyl-CoA O-methyltransferase 4
CcoAOMT5	caffeoyl-CoA O-methyltransferase 5
CHI-A	CHALCONE ISOMERASE-A; encodes chalcone isomerase
CHS-A	CHALCONE SYNTHASE-A; encodes chalcone synthase
CHS-J	CHALCONE SYNTHASE-J; encodes chalcone synthase
CNL1	Trans-cinnamate:CoA ligase 1
DFR	dihydroflavonol 4-reductase
DIF-F	DIFFERENTIAL-F; encodes a cytochrome B5 protein
DPL	DEEP PURPLE; encodes an R2R3-MYB transcription factor
EGS	eugenol synthase
EOBI	Emission of benzenoids I
EOBII	Emission of benzenoids II
F3H	flavanone 3-hydroxylase
FA	Fading locus
FLS	flavonol synthase
HF1 (F3’5’H)	HYDROXYLATION AT FIVE-1; encodes flavonoid 3’5’-hydroxylase
HF2 (F3’5’H)	HYDROXYLATION AT FIVE-2; encodes flavonoid 3’5’-hydroxylase

HT1 (F3'H)	HYDROXYLATION AT THREE-1; encodes flavonoid 3'-hydroxylase
IGS	isoeugenol synthase
LAR1	LIGHT AREAS1; encodes an R2R3-MYB transcription factor
MIXTA	encodes an R2R3-MYB transcription factor
MF1 (3'5'AMT)	METHYLATION AT FIVE-1; encodes 3'5'AMT
MF2 (3'5'AMT)	METHYLATION AT FIVE-2; encodes 3'5'AMT
MT (3'AMT)	METHYLATION AT THREE; encodes 3'AMT
MYB27	encodes an R2R3-MYB transcription factor
MYB4	encodes a MYB-like transcription factor
MYBX	encodes an R3-MYB repressor protein
MYB-FL	R2R3-MYB transcription factor controlling UV pigmentation
OS	ocimene synthase
PAAS	phenylacetaldehyde synthase
PAL-A	PHENYLALANINE AMMONIA LYASE-A; encodes phenylalanine ammonia lyase
PAL-B	PHENYLALANINE AMMONIA LYASE-B; encodes phenylalanine ammonia lyase
PAL-C	PHENYLALANINE AMMONIA LYASE-C; encodes phenylalanine ammonia lyase
PH1	magnesium-transporting ATPase
PH3	encodes a WRKY transcription factor
PH4	encodes an R2R3-MYB transcription factor affecting vacuolar acidification
PH5	plasma membrane ATPase
PhLHY	*Petunia hybrida* LATE ELONGATED HYPOCOTYL; encodes a circadian clock gene
PHZ	PURPLE HAZE; encodes an R2R3-MYB transcription factor
PIN	PIN-FORMED family of auxin efflux transporters
RAN1	Copper-transporting ATPase RAN1
RT	3-rhamnosyl transferase
SAND	SAND family protein
ODO1	ODORANT1; encodes an R2R3-MYB transcription factor
YUP	YELLOW UPPER locus

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Chapter 1

Introduction

Understanding the genetic basis of speciation

To this day understanding the origin of species remains one of the fundamental questions in biology. The vast diversity of organisms found in nature have evolved due to a process termed speciation. Darwin viewed speciation as the process by which species entered new ecological niches and adapted to them, ultimately leading to the separation of the two species (Darwin, 1859). In his theory of natural selection he emphasized the gradual nature of this speciation process in populations. However, Darwin's ideas on speciation were made in the absence of genetics. It was only later that genetics and the concept of speciation were united and Dobzhansky's work gave rise to the modern understanding of speciation (Dobzhansky, 1937; Coyne, 2016). He recognized that for speciation to occur, barriers are needed that provide reproductive isolation and allow species to diverge through the prevention of gene flow, emphasizing the importance of isolating mechanisms.

There are two main groups of speciation barriers that can occur: prezygotic and postzygotic barriers (Fig. 1.1, adapted from Rieseberg and Blackman, 2010). Prezygotic barriers refer to barriers that occur before fertilization while postzygotic barriers describe barriers occurring after fertilization. Prezygotic barriers include but are not limited to habitat isolation, temporal isolation and behavioral isolation (Rieseberg and Blackman, 2010). Postzygotic barriers can include hybrid sterility, inviability or breakdown. In plants specifically, prezygotic barriers can further be separated into pre- and post-pollination barriers, that describe whether a barrier occurs before or after pollen is transferred to the stigma (Rieseberg and Blackman, 2010). The influence of both pre- and postzygotic barriers can be measured directly in the field or through experimental manipulation, which can then be used to illustrate the impact of the different isolating barriers. Reproductive isolation is often not only caused by a single pre- or postzygotic barrier isolating two species from one another, but involves a number

of these barriers and their interactions (Coyne and Orr, 2004; Rieseberg and Willis, 2007a). Isolation barriers act in a linear order, with prezygotic barriers occurring before postzygotic barriers. Due to this linearity, prezygotic barriers act early in the life cycle of an organism and will contribute more to overall isolation if multiple barriers are present. Through their early influence they can cause the strongest obstruction to gene flow and potentially prevent unfavorable costly mating combinations (Jiggins and Mallet, 2000; Jiggins et al., 2001). In flowering plants specifically, reproductive isolation is often caused by prezygotic isolation mechanisms rather than postzygotic barriers (Ramsey et al., 2003; Kay, 2006; Rieseberg and Willis, 2007b; Lowry et al., 2008; Widmer et al., 2009; Dell'Olivo et al., 2011; Baack et al., 2015).

Figure adapted from Fig. 1 of Rieseberg and Blackman, 2010.
https://doi.org/10.1093/aob/mcq126

Figure 1.1: Reproductive isolation barriers occur in a linear order (figure adapted from Rieseberg and Blackman, 2010). Different isolating mechanisms occur at multiple stages throughout the life cycle. Prezygotic barriers describe barriers that occur before fertilization while postzygotic barriers occur after fertilization. Prezygotic barriers can be further divided in two categories depending on whether pollen has been transferred to the stigma (post-pollination) or not (pre-pollination). Figure was created using Biorender.com.

To further understand the impact of different reproductive barriers on speciation, we need to determine the molecular-genetic basis of these barriers. The first step in a speciation process is local adaptation and the genetic basis of adaptation was described in the classical model by Fisher in 1930. In this model he stated that speciation occurred due to changes in many genes and the accumulation of these over time (Fisher, 1930; Orr,

2005a). Each of these genes is considered to have a small effect. These small mutations allow descendants to gradually diverge from the parental population without removing it far from its fitness optimum. Large effect mutations are not considered beneficial for adaptation in this model as they remove a species far from its fitness optimum, most likely producing phenotypes with decreased fitness (Fisher, 1930; Orr, 2005a). There is accumulating experimental evidence in different systems demonstrating a complex genetic basis of variation in traits with many loci involved (Chan et al., 2010; Turchin et al., 2012; Kooke et al., 2016; Guo et al., 2018; Martin et al., 2019; Sohail et al., 2019; Kautt et al., 2020).

However, this gradual process also comes with challenges. Fisher's model assumed that adaptation occurred through the appearance of *de novo* mutations and did not include standing genetic variation (Fisher, 1930; Orr, 2005a). Also, the fitness optimum towards which species were adapting to is stationary in his model, not moving during a bout of adaptation (Fisher, 1930; Orr, 2005a). Under allopatric speciation (speciation occurring through geographical isolation) this mechanism of adaptation including many genes is feasible since an instant cessation to gene flow is caused by the geographic barrier and populations can no longer exchange genetic material (Coyne and Orr, 2004). However, in sympatry (speciation occurring without geographical barriers) gene flow is ongoing with recombination having homogenizing effects on populations, making sympatric speciation difficult to connect with gradual change.

Fisher's model was challenged by Orr's theory in 1998 who proposed that mutations of large effect are not always deleterious but can be beneficial to the speciation process. A continuum of mutations with large to small effects was proposed to be the driver of speciation (Orr, 1998). Based on theoretical work, having few mutations of large effect that quickly create large phenotypic differences between the populations can drive speciation when selection is strong (Orr and Coyne, 1992; Orr, 2005a). The genetic basis of individual components of reproductive isolation has been studied in various systems and mutations of large effect have been shown to play an essential role in the emergence of new species (Doebley, 2004; Hoekstra et al., 2006; Rieseberg and Blackman, 2010; Presgraves, 2010; Nadeau et al., 2016; Todesco et al., 2020). To gain insight into these ongoing speciation processes, contemporary research has therefore focused on

identifying such genes that are functionally differentiated between nascent species. These genes of large effect have been coined "speciation genes".

Orr defined speciation genes as genes contributing to reproductive isolation (Orr, 2005b). This contribution can either be the initial mechanism leading to isolation or mechanisms that arise later during speciation. Rieseberg and Blackman (2010) use a similar definition in which a speciation gene is a "gene that contributes to the splitting of two lineages by reducing the amount of gene flow between them". In both definitions, speciation genes need to contribute to isolation between species by influencing reproductive barriers. Rieseberg and Blackman (2010) specifically outline four requirements that should ideally be met to obtain a comprehensive analysis of a speciation gene: (1) Both the gene and mutations underlying reproductive isolation should be functionally characterized, (2) Phylogenetic and geographical distribution of the different alleles affecting reproductive isolation should be studied, (3) Selection should be assessed through evolutionary analyses and (4) Field studies in natural populations should be carried out to examine the effects of allelic variants.

However, I argue that although fulfilling all these requirements would be ideal, they are not all required to be able to classify a gene as a speciation gene, since several convincing examples of speciation genes have been demonstrated without all four requirements being met (Des Marais and Rausher, 2010; Byers et al., 2014; Amrad et al., 2016; Sheehan et al., 2016; Esfeld et al., 2018). The first requirement is highly important to define a speciation gene as such, since this lays the fundamental work of describing the gene and mutations causing differences between species. Once the alleles of the speciation gene have been determined, phylogenetic analyses, as proposed in requirement 2, determining how these mutations are distributed between species are critical to demonstrate allelic differences between species. However, geographical distribution of the alleles may be less important compared to previous analyses, as this paints a picture of the extant distribution of alleles and is not necessarily representative of the distribution at speciation. This is also a concern for requirement 3, as selection should ideally be assessed at the moment of speciation, which can never be fully done in hindsight. However, ongoing mechanisms strengthening the reproductive barriers in existing species may be equally as important for speciation to remain intact. Combining analyses

of ongoing selection with patterns of previous selection determined through molecular evolution methods on population level sampling to infer what alleles were under selection during the speciation process would paint a full picture of past and present selection. Assessing the selective effects of the alleles in natural populations as proposed in requirement 4, is highly relevant as this demonstrates whether the differences determined under laboratory conditions can also be observed in natural populations. In this thesis I therefore consider speciation genes to be genes who fit both Orr's and Rieseberg/Blackman's definitions, and for which substantial work has been done to satisfy at least two of the ideal requirements of functional, organismal and population-level studies.

Speciation genes can cause barriers to gene flow at pre- or postzygotic stages. As reproductive isolation in flowering plants is often caused by prezygotic isolation mechanisms rather than postzygotic barriers due to reproductive isolation barriers occurring in a linear order if multiple barriers are present (Ramsey et al., 2003; Kay, 2006; Rieseberg and Willis, 2007b; Lowry et al., 2008; Widmer et al., 2009; Dell'Olivo et al., 2011; Baack et al., 2015), many speciation genes involved in prezygotic barriers have been detected. Most major effect genes influencing prezygotic barriers in plants that have been described to date, affect pre-pollination processes, mainly by altering processes such as floral color display (Quattrocchio et al., 1999; Schwinn et al., 2006; Hoballah et al., 2007; Des Marais and Rausher, 2010; Yuan et al., 2016), floral scent emission (Klahre et al., 2011; Amrad et al., 2016) or flowering time (time period during which a plant produces flowers) (Wang et al., 2006; Salomé et al., 2011; Woods et al., 2017; Taylor et al., 2019). In the genes influencing floral color display multiple trends can be observed (Rieseberg and Blackman, 2010). The first observed trend being that variation in flower color contributes to prezygotic isolation and it appears that similar pathways and thus genes may be responsible for reproductive isolation. For example, shifts in visible floral color are often due to changes in the flavonoid biosynthetic pathway producing anthocyanins (Hoballah et al., 2007; Des Marais and Rausher, 2010; Smith and Rausher, 2011; Esfeld et al., 2018; Berardi et al., 2021). The second observed trend is that losses and transitions in visible floral color are caused by different groups of genes. While transitions in visible floral color are often caused by biosynthetic genes (Hoshino et al., 2003; Zufall and Rausher, 2004; Streisfeld and Rausher, 2009a; Des Marais and Rausher, 2010; Hopkins

and Rausher, 2011; Smith and Rausher, 2011; Wessinger and Rausher, 2012, 2014), genes involved in transcriptional regulation lead to loss of color (Quattrocchio et al., 1999; Durbin, 2003; Schwinn et al., 2006; Hoballah et al., 2007; Streisfeld and Rausher, 2009b; Yuan et al., 2013). Prezygotic barriers therefore offer an appealing system to determine the genes that are functionally different between species.

Studying reproductive isolation through pollination syndrome transitions

An ideal case to test the genetic basis of reproductive barriers is animal-mediated pollination, one of the most important types of prezygotic barriers in plants. As plants are sessile they must rely on vectors to effectively disperse pollen or seeds. Many plant species have therefore developed a mutually beneficial relationship with pollinators to reproduce successfully. Depending on the geographical region, between 78% to 94% of flowering plants are pollinated by animals (Ollerton et al., 2011) and animal-mediated pollination has been shown to have a direct impact on species diversity (Sapir and Armbruster, 2010; Schiestl and Johnson, 2013; Van der Niet et al., 2014) while also being widely accepted as a driving force of radiation in angiosperms (Grant and Grant, 1965), demonstrating its importance for plant reproduction. Pollinators transport pollen between different plants, allowing for outcrossing to occur and providing the plants with pollination services while in return gathering rewards such as nectar and/or pollen. To attract specific pollinators, plants evolved floral displays matching the preference of the pollinators to advertise their potential rewards. This specific pollinator attraction has been shown to contribute to the isolation and speciation of outcrossing species (Van der Niet et al., 2014). As pollinators are directly linked to the plant's reproductive success, they can exert selection on floral traits, such as color, shape and scent. Depending on the pollinator assemblage these selection pressures can diverge, resulting in phenotypic differences in floral traits. Using closely related plant species that have evolved from a common ancestor and are visited by different pollinators, has allowed the role of pollinators in speciation to be demonstrated (Bradshaw and Schemske, 2003; Ramsey et al., 2003; Castellanos et al., 2006; Kay and Sargent, 2009).

Pollination syndromes are specific suites of floral traits that have evolved to attract a specific group of pollinators (Faegri and Pijl, 1979; Fenster et al., 2004). It was Darwin,

who first thought about specific plant-pollinator interactions during his trips to the Galapagos Islands. He observed that the nectar containing spur of an orchid was extremely long and predicted that its pollinator must be a moth with a proboscis long enough to reach the nectar (Darwin, 1862). It would take many years for Darwin's prediction to be verified and through field observations it is now known that the moth *Xanthopan morganii praedicta* exclusively pollinates this orchid exhibiting a highly specialized pollination syndrome (Arditti et al., 2012). Although an extreme example, this highlights the fact that pollinator groups are often attracted to particular floral traits. When comparing species with different pollination syndromes, there are three main categories of traits affecting pollination success to consider (Bradshaw et al., 1995). Attraction traits help in guiding pollinators to specific flowers and include color, fragrance, flower shape and size. Rewarding traits are generally available to the pollinator after contact with the flower, and can include nectar volume, nectar composition and pollen quantities. Efficiency traits affect how efficient animal-mediated pollen transfer can occur and consist of traits influencing reproductive organ morphologies.

For pollination to be successful, many different signals act at once, either attracting or deterring pollinators. The precise contribution of a single trait is often difficult to entangle as traits are linked and interact with one another. Floral displays involving complex signals through different sensory modes may help for recognition where some traits serve as a backup under circumstances when other traits fail (Lawson et al., 2017). The pollination syndrome concept therefore implies that pollinators are classified into functional groups (Fenster et al., 2004). Examples of such groups can be pollen collecting bees or long-tongued moths. Pollinators clustered together in these functional groups behave similarly which leads to similar selection pressures being exerted by them on the floral displays of plants (Fenster et al., 2004). These similar selection pressures should then lead to similar floral traits being presented to attract a specific functional group of pollinators (Fenster et al., 2004). In cases where the pollinator and flower have evolved very specifically, floral rewards may not be accessible to other pollinator groups (e.g. long tubes where a long tongue or proboscis is needed to reach the nectar). However, plants are often not only visited by one pollinator, seemingly contradicting the pollination syndrome theory.

Specialization in terms of pollinator interactions describes the concept where a plant and pollinator have adapted to one other and the specialist pollinator is able to access the resources the plant provides through this specific interaction (Armbruster, 2017). A specialist pollinator should only visit very few plant species to which it is strongly adapted to, increasing the probability of conspecific pollen being deposited efficiently (Maldonado et al., 2013). The concept of specialization was thought to be ideal as a single pollinator species specialized to a plant species should be the most efficient pollinator and therefore maximize reproductive success (Fenster et al., 2004; Stebbins, 1970). However, there are also disadvantages that come with such specialization. One important aspect to consider is the cost of an additional pollinator. If selection for certain features favors attracting a new group of pollinators without losing an old one (Aigner, 2001), the visitation by multiple pollinators comes with no additional cost to the plant (Fenster et al., 2004), e.g. if a nocturnally pollinated flower stays open during the day and can be visited by nocturnal and diurnal pollinators without any competition between the functional groups. Therefore if a plant remains highly specialized to a single pollinator group this may exclude other functional groups from visiting their flowers, possibly decreasing the pollination services they are receiving. Also under changing environmental conditions, having multiple pollinator groups that can offer pollination services is helpful if shifts in the pollinators present in the environment occur.

The opposing view to specialization is generalization, a concept which describes that pollinators are functionally equivalent (Gómez, 2002). If numerous animal species are able to pollinate equally well and select for the same floral traits, having a more generalized pollination syndrome may be beneficial to the plant rather than being adapted to a single pollinator group. However, having a generalized pollination syndrome is also not advantageous under all circumstances. If the plant phenotype attracts a wide range of pollinators, but only a few are effective, this may lead to a decrease in reproductive success of the plant as the ineffective pollinators can deplete the rewards the plant offers. The wider the range of pollinators that are being attracted, the more likely it is that pollinators with positive, neutral and negative effects will visit (Padyšáková et al., 2013). Pollination syndromes can therefore be described as a spectrum, ranging from generalist plants with flowers attracting many different

pollinator groups to plants with specific flowers developed for very specialized pollinators (Fenster et al., 2004).

The concept of pollination syndromes not only provides a theoretical framework, but is particularly useful for understanding floral diversification (Kingston and Quillan, 2000; Fenster et al., 2004; Ollerton et al., 2009; Danieli-Silva et al., 2012). Adaptations to pollinator types are fundamentally important for the divergence of floral phenotypes (Raven, 1972; Thomson and Wilson, 2008; Abrahamczyk and Renner, 2015; Cardona et al., 2020) and such shifts have occurred surprisingly frequently (Knapp, 2010). An example of a system where key floral traits affecting pollinator behavior have caused reproductive isolation and adaptation is the monkeyflower (*Mimulus*). The genetic basis of differences in pollination syndromes between *Mimulus* species has been the focus of many studies due to the wide array of phenotypic, ecological and genomic diversity in this system. For instance, pigments responsible for differences in floral color, such as carotenoids, were attributed to a major quantitative trait locus (QTL) while flower size traits and nectar volume were linked to at least one large effect QTL, suggesting that these loci may have contributed to the shift in pollinators observed in these sympatric *Mimulus* species (Bradshaw et al., 1995, 1998). In allele swapping experiments, the authors demonstrated that changes in the *YUP* pigmentation locus lead to strong phenotypic alterations affecting pollinator preference (Bradshaw and Schemske, 2003). Combining the genetic basis of pollination syndromes with pollinator observations in this system has allowed a better understanding of floral diversification. Another example of a system where key floral traits affect pollinator behavior and have led to reproductive isolation and adaptation is the genus *Petunia* (Gübitz et al., 2009; Dell'Olivo et al., 2011; Fregonezi et al., 2013; Vandenbussche et al., 2016). Here different pollination syndromes and therefore selection for different pollinators has ultimately led to floral diversification (Fregonezi et al., 2013).

The genus *Petunia* as a model system for floral evolution

The genus *Petunia* belongs to the *Solanaceae* family and originates from South America, (Stehmann and Semir, 2005; Stehmann et al., 2009; Reck-Kortmann et al., 2014). Wild *Petunia* species offer an ideal system to study the evolution of plants and pollinators

(Hoballah et al., 2007; Esfeld et al., 2018; Yarahmadov et al., 2020; Berardi et al., 2021). The three major pollination syndromes that can be found in *Petunia* (bee, hawkmoth and hummingbird syndrome) all display differences in the categories of traits affecting pollination success. Bee pollinated flowers generally display an open morphology (Stuurman et al., 2004) that allows nectar to be accessed by crawling inside the flower. These pollinators have a diurnal activity (Hoballah et al., 2007) and colors of the *Petunia* flowers range from pink to purple to attract bee pollinators (Stehmann et al., 2009; Hermann and Kuhlemeier, 2011; Dell'Olivo and Kuhlemeier, 2013). The hawkmoth pollination syndrome is typically associated with white flowers and long narrow corolla tubes that are UV absorbent (Sheehan et al., 2016). The flowers emit fragrant, volatile compounds at night (Hoballah et al., 2005) and produce large amounts of nectar (Stuurman et al., 2004). Flowers displaying a hummingbird syndrome are red with a long, narrow corolla tube (Rodríguez-Gironés and Santamaría, 2004). The anthers and stigmas are exserted with the flower's petals reflexed (Bradshaw et al., 1995). As in the hawkmoth syndrome, large amounts of diluted nectar are produced (Bradshaw et al., 1995).

Low intra- and interspecific genetic diversity among the species investigated, suggest that *Petunia* species have diverged recently, approx. 2.85 – 1.3 million years ago (Lorenz-Lemke et al., 2010; Särkinen et al., 2013). This recent diversification has made the phylogeny of the *Petunia* genus difficult to resolve (Reck-Kortmann et al., 2014). Based on molecular phylogenetic analyses *Petunia* can be categorized into two major clades: the short tube and long tube clade (Fig. 1.2; figure adapted from Reck-Kortmann et al., 2014 and Esfeld et al., 2018). The short tube clade includes 14 purple flowered and bee-pollinated species, while the long tube clade comprises six species. Unlike the short tube clade, the long tube clade presents different pollination syndromes (hawkmoth, hummingbird and bee pollinated) (Stehmann, 1987; Ando, 2001; Stehmann and Semir, 2005; Lorenz-Lemke et al., 2006; Reck-Kortmann et al., 2014; Rodrigues et al., 2018a). The ancestral state of *Petunia* was most likely a short corolla tube with bee pollination floral syndrome, much like the extant species in the short tube clade (Ando et al., 2005; Kulcheski et al., 2006; Esfeld et al., 2018). While the separation of the long tube and short tube clade is strongly supported by published phylogenies, the precise phylogenetic relationships of the species in the long tube clade is more difficult to resolve (Reck-Kortmann et al., 2014; Esfeld et al., 2018). Specifically the placement of *Petunia secreta*

and *Petunia exserta*, the two colored species, has differed between phylogenies (Fig. 1.2). The most recent study robustly places *P. secreta* and *P. exserta* as sister species to the white-flowered *Petunia axillaris* (Fig. 1.2 B, Esfeld et al., 2018). Advantages of this newer phylogeny were the inclusion of genome-wide datasets as well as analyses performed using two different approaches to construct the species tree. Both approaches yielded the same topology (Esfeld et al., 2018), giving strong support to the placement of the colored species as a species pair that is sister to *P. axillaris*. The study presented here focuses on four main species of the *Petunia* clade, that display all three major pollination syndromes: *Petunia integrifolia ssp. inflata* from the short tube clade as well as *P. axillaris*, *P. exserta* and *P. secreta* all from the long tube clade (Fig. 1.3).

Panel A adapted from Fig. 1 of Reck-Kortmann et al., 2014.
https://doi.org/10.1016/j.ympev.2014.08.022

Panel B adapted from Fig. 2A of Esfeld et al., 2018.
https://doi.org/10.1016/j.cub.2018.10.019

Figure 1.2: Phylogenetic relationship of *Petunia* species (adapted from Reck-Kortmann et al., 2014 and Esfeld et al., 2018). The *Petunia* genus can be divided into two major clades based on corolla tube morphology: the short tube (shown in purple) and long tube clade (depicted in gray). (A) The short tube clade comprises many species with similar morphology, all bee pollinated while the long tube clade consists of species with different pollination syndromes. *P. axillaris* and *P. secreta* are shown as sister species to *P. exserta*. *Calibrachoa*, a sister genus, is shown as the outgroup. (B) The most recent study robustly places *P. secreta* and *P. exserta* as sister species to *P. axillaris*. The tree was rooted with the short tube clade species *P. inflata*.

P. integrifolia

The morphology of *P. integrifolia* can be used as a representative for all 14 species of the short tube clade. It exhibits a typical bee pollination syndrome with purple colored, UV reflective, short tubed flowers that are self-incompatible (Reck-Kortmann et al., 2014)

and emits only a single scent volatile (benzaldehyde) (Hoballah et al., 2005; Amrad et al., 2016). Compared to hawkmoth or hummingbird pollinated species, *P. integrifolia* produces low amounts of nectar and its main pollinators are small bees (Fig. 1.3 A)(Gübitz et al., 2009; Brandenburg et al., 2012a). Although its main pollinators are bees, this species is also visited by diurnal butterflies (Hoballah et al., 2007). Pollination only occurs during the day and not at night (Hoballah et al., 2007). *P. integrifolia* can be found in southern Brazil in habitats ranging from rock crevices and disturbed sites to sandy soils and can produce fertile offspring when artificially crossed with species of the long tube clade, such as *P. axillaris,* but does not appear to hybridize when occurring in sympatry (Ando et al., 2005; Dell'Olivo et al., 2011). Geographic isolation was determined to be the most important factor isolating the short tube clade from *P. axillaris* (Dell'Olivo et al., 2011). Work in this thesis specifically focuses on *P. integrifolia ssp. inflata* (hereafter referred to as *P. inflata*) as a representative of the short tube clade.

P. axillaris

P. axillaris belongs to the long tube clade and has white colored flowers that have been found to be both self-incompatible and self-compatible depending on the population (Kokubun et al., 2006; Turchetto et al., 2015a). The floral limbs are UV absorbent, caused by flavonol accumulation (Sheehan et al., 2016). Contrary to the short tube clade, they emit scent volatiles at dusk and at night to attract pollinators (Hoballah et al., 2005). The fragrant bouquet of *P. axillaris* is dominated by methylbenzoate, benzaldehyde and benzylalcohol (Hoballah et al., 2005). The flowers produce large amounts of diluted nectar (Lorenz-Lemke et al., 2006; Brandenburg et al., 2009) and are visited by different hawkmoth species at night (Fig. 1.3 B) (Venail et al., 2010; Dell'Olivo et al., 2011; Klahre et al., 2011). Although *P. axillaris* displays a typical hawkmoth pollination syndrome, visitation during the day by bees and beetles was also observed (Hoballah et al., 2007; Dell'Olivo et al., 2011). Its habitat is similar to that of *P. inflata* and includes rock crevices, disturbed sites and sandy soils (Dell'Olivo et al., 2011). *P. axillaris* is occasionally found in sympatry with *P. inflata* from the short tube clade, but also with other species from the long tube clade, such as *P. secreta* and *P. exserta* (Turchetto et al., 2015b; Rodrigues et al., 2018a).

P. exserta

This species exhibits a typical hummingbird pollination syndrome, with red flowers, a long tube and copious amounts of nectar (Watanabe et al., 2001; Lorenz-Lemke et al., 2006; Stehmann et al., 2009). The anthers and stigmas of *P. exserta* are exserted (Lorenz-Lemke et al., 2006; Stehmann et al., 2009) a trait also typically associated with hummingbird pollination and the flowers are self-compatible (Caballero-Villalobos et al., 2021). Accordingly, the primary pollinator was observed to be hummingbirds (Fig. 1.3 C) (Stehmann, 1987). *P. exserta* can be found in rocky shelters and shaded spots of sandstone towers (Caballero-Villalobos et al., 2021).

P. secreta

P. secreta displays long tubed, purple colored flowers that are self-compatible (Turchetto et al., 2016; Rodrigues et al., 2018b). In contrast to *P. axillaris*, no scent volatiles are emitted and low amounts of nectar are produced (Rodrigues et al., 2018a). *P. secreta* can be found in very restricted geographical region in low elevation mountain ranges in the Sierra do Sudeste of Brazil (Stehmann et al., 2009; Rodrigues et al., 2018b). Two lineages in slightly different environments have been detected thus far (Turchetto et al., 2016). Halictid bees of the genus *Pseudagapostemon,* a communal nesting solitary bee, were observed to be the primary pollinator (Fig. 1.3 D) (Rodrigues et al., 2018a) although *P. secreta* does not display all traits of a typical bee pollination syndrome with its long tube. Visitations to *P. secreta* by *Pseudagapostemon* are infrequent (38 visits during 225 h) pointing towards visitation by other pollinators possibly helping maintain these populations (Rodrigues et al., 2018a).

Panel A adapted from Fig. 2.1 D of Gübitz et al., 2009.
https://doi.org/10.1007/978-0-387-84796-2

Panel B and C photos from Alexandre Dell'Olivo.

Panel D adapted from Fig. 1C of Rodrigues et al., 2018a.
https://doi.org/10.1093/aobpla/ply057

Figure 1.3: *Petunia* species display different pollination syndromes. (A) *P. integrifolia*, representative for the short tube clade, is visited by a solitary bee (Photo: Gübitz et al., 2009). (B) *P. axillaris*, a member of the long tube clade, is pollinated by a hawkmoth. (Photo: Alexandre Dell'Olivo). (C) Hummingbird visiting a *P. exserta* flower. (Photo: Alexandre Dell'Olivo). (D) *P. secreta* flower being visited by a solitary bee (Photo: Rodrigues et al., 2018a).

Petunia species vary in their range distributions, with some species, such as the white-flowered *P. axillaris*, showing a wider distribution range across different countries and others, such as the endemic and purple-flowering *P. secreta*, existing in only a single restricted region (Reck-Kortmann et al., 2014). Certain species, such as the white-flowered *P. axillaris* and red-flowered *P. exserta*, occur in sympatry and plants with intermediate floral phenotypes and morphological characteristics have been found in these natural hybrid zones (Segatto et al., 2014; Turchetto et al., 2015b, 2019; Caballero-Villalobos et al., 2021). *P. secreta* also occurs in the same geographical region as *P. axillaris* but its distribution range is much more restricted (Turchetto et al., 2016; Rodrigues et al., 2018b). Hybrids of these two species with intermediate morphologies have also been observed in these areas (M. Lüthi, A. Berardi, personal observation). The divergence of the *Petunia* genus in southern Brazil is remarkable for the restricted range of most species (Lorenz-Lemke et al., 2010; Särkinen et al., 2013). These overlapping regions with

endemic species have proven to be highly important for studying diversification and speciation processes as these are often still ongoing.

The emergence of *Petunia* as a model system for molecular genetics

Not only is the *Petunia* genus ideal for studying speciation processes and plant pollinator interactions but it has emerged as a model system for molecular genetics (Gerats and Vandenbussche, 2005; Vandenbussche et al., 2016) with its main research focus lying on *Petunia hybrida*. This cultivar is the result of crosses between *P. axillaris* and multiple short tubed purple-flowered *Petunia* species (Segatto et al., 2014; Wijsman, 1982) and has allowed many processes to be investigated (2009). These processes include post-transcriptional gene silencing (Napoli et al., 1990; van der Krol et al., 1990; Atkinson et al., 1998; Sijen et al., 2001; Ban et al., 2019), flower development (van der Krol et al., 1993; Morel et al., 2019; Brandoli et al., 2020), studies on self-incompatibility (Robbins, 2000; Kubo et al., 2015; Williams et al., 2015; Sun et al., 2018) as well as extensive research on anthocyanin pigmentation (Napoli et al., 1990; Quattrocchio et al., 1999; Koes et al., 2005; Albert et al., 2009, 2011, 2014). A combination of aspects in *Petunia* make it an intriguing model system, providing advantages over other model systems, such as *Arabidopsis* (Gerats and Vandenbussche, 2005). For instance compared to *Arabidopsis* where it is difficult to obtain progenies from crosses between different species, *Petunia* allows these crosses between species to be performed fairly easily. Additionally *Petunia* occupies a wide range of ecological niches, that cannot be found in *Arabidopsis* (Gerats and Vandenbussche, 2005). Further aspects that make the *Petunia* system intriguing is the possibility of easy asexual propagation and a large set of mutants, mainly acquired through transposon insertions, that is available. Also, the set of genes that is functionally as well as molecularly characterized is extensive and keeps increasing. Advances in sequencing analyses of the complete genome of different *Petunia* species as well as RNAseq approaches in recent years have helped further establish *Petunia* as a model system (Vandenbussche et al., 2016).

Floral color and its impact on pollinator attraction

The advertisement of a flower to its potential pollinator occurs through attraction traits, such as floral color and scent or a combination of the two. Floral pigmentation is used to

advertise potential rewards to pollinators and ideally attract the most efficient pollinator to the plant to maximize pollination success. The genetic basis of floral pigmentation has therefore been a target of many research studies (Bradshaw et al., 1995, 1998; Bradshaw and Schemske, 2003; Hoballah et al., 2007; Sheehan et al., 2016) and transitions in floral color have played an important role in the reproductive isolation of species (Hoballah et al., 2007; Cardona et al., 2020). Such transitions have occurred often in the *Solanaceae*, for example red floral color associated with bird-type pollination syndromes having evolved individually at least ten times (Knapp, 2010).

Color transitions are mainly controlled through mutations in biosynthetic genes, whether this be through mechanisms of inactivation, deletion or downregulation (Hoshino et al., 2003; Zufall and Rausher, 2004; Des Marais and Rausher, 2010; Smith and Rausher, 2011; Hopkins and Rausher, 2012; Wessinger and Rausher, 2012; Wessinger et al., 2014). The loss of visible color specifically, has predominantly occurred through mutations in regulatory genes of biosynthetic pathways as described in some well-known systems such as *Petunia* and *Mimulus* (Quattrocchio et al., 1999; Schwinn et al., 2006; Hoballah et al., 2007; Streisfeld and Rausher, 2009a; Yuan et al., 2013). Although losses of floral pigmentation have been documented frequently in the angiosperms, re-gaining color is also possible, as has been described in sister species *P. secreta* (Esfeld et al., 2018) and *P. exserta* (Berardi et al., 2021).

While floral pigmentation is extremely important for pollinator attraction, it can also serve other purposes for plants. High accumulations of pigments in flowers and leaves provide protection from harmful UV radiation, drought or heavy metals (Middleton and Teramura, 1993; Koes et al., 1994; Chalker-Scott, 1999; Gould, 2004). Resistance to biotic stressors, such as herbivores and pathogens, can also be conferred through pigmentation (Gould, 2004). Certain pigments can even help stabilize PIN efflux complexes that regulate polar auxin transport (Teale et al., 2021), highlighting the many important facets of floral color.

Production of floral color through the flavonoid biosynthetic pathway

When analyzing floral color in *Petunia*, two main categories of floral pigments can be detected: visible and UV color pigments. Both are produced from common precursors in

the flavonoid biosynthetic pathway and *Petunia* species show the presence of flavonols and anthocyanins in their flower petals (Sheehan et al., 2016; Esfeld et al., 2018). Anthocyanins are responsible for the visible color pigmentation while flavonols determine UV color appearance. Figure 1.4 shows an overview of the *Petunia* flavonoid biosynthetic pathway (adapted from Esfeld et al., 2018 and Berardi et al., 2021) and displays precursors that are shared by both branches (flavonols and anthocyanins) of the pathway. The last common precursors to flavonols and anthocyanins are the dihydroflavonols DHK, DHQ and DHM, which are modified by pathway branching enzymes F3'H and F3'5'H that have hydroxylating functions. At every branch in this stage of the pathway, FLS and DFR can compete for the dihydroflavonol substrates. A functioning FLS enzyme can use these precursors to produce the flavonols kaempferol, quercetin and myricetin (mono, di-, and trihydroxylated in ascending order). However, myricetin levels are strongly reduced in *Petunia* as FLS has low activity on DHM (Holton et al., 1993). DFR requires the sequential action of ANS to produce the anthocyanidins cyanidin and delphinidin (dihydroxylated and trihydroxylated, respectively), which range in color from magenta to blue/purple. *Petunia* species are not able to produce the red monohydroxylated pelargonidin pigment as the enzyme DFR is unable to accept DHK as a substrate (Johnson et al., 2001). Further modifications of cyanidin and delphinidin by subsequent biosynthetic enzymes (3GT, ART, AAT and 5GT, 3'AMT and 3'5'AMT) lead to the production of the three methylated anthocyanins peonidin (red-magenta), petunidin and malvidin (both magenta-blue).

Figure adapted from Fig. 1 of Esfeld et al., 2018 and Fig. 1A of Berardi et al., 2021.
https://doi.org/10.1016/j.cub.2018.10.019
https://doi.org/10.1093/plcell/koab114

Figure 1.4: Flavonoid biosynthetic pathway in *Petunia* (figure adapted from Esfeld et al., 2018 and Berardi et al., 2021). Flavonols and anthocyanins are produced through individual branches of the flavonoid biosynthetic pathway in *Petunia*. Enzymes needed for individual steps are depicted above or beside the respective arrow. Substrates are highlighted in bold font. Transcription factors *AN2* and *MYB-FL* (highlighted in light blue) activate *DFR* and *FLS* respectively. Light gray enzymes and arrows depict reactions that do not occur in *Petunia* as the enzyme DFR does not accept DHK as a substrate (Johnson et al., 2001) and the enzyme FLS has low activity on DHM (Holton et al., 1993). See abbreviations for enzyme names. Figure was created using Biorender.com.

Various plant taxa show altered anthocyanin production and floral pigmentation through anthocyanin-regulating transcription factors (Bovy et al., 2002; Hoballah et al., 2007; Albert et al., 2011). The visible pigmentation pattern in *Petunia* specifically is controlled through closely related transcription factors *AN2, DPL, PHZ* and *AN4* (Quattrocchio et al., 1999; Hoballah et al., 2007; Albert et al., 2011). These transcription factors all belong to the family of the R2R3-MYB transcription factors and interact with basic helix-loop-helix (bHLH) and WD-repeat (WDR) partners to form protein triplexes that activate pigmentation biosynthesis (Koes et al., 2005; Albert et al., 2011). The bHLH factor *AN1* and WDR partner *AN11*, together with the aforementioned four MYB transcription factors, regulate the different areas of pigmentation in the *Petunia* flower as shown in Fig. 1.5 (figure adapted from Albert et al., 2011). The flower tube and anther coloration is controlled by *AN4* + *AN1* + *AN11* while floral bud blushing is regulated by the presence of the same bHLH and WDR partners forming a complex with the transcription factor *PHZ* (Albert et al., 2011). *DPL* + *AN1* + *AN11* is responsible for the visible pigmentation of

flower tube venation while the triple complex *AN2* + *AN1* + *AN11* controls the pigmentation of the petal limb (Quattrocchio et al., 1999; Hoballah et al., 2007; Albert et al., 2011).

Figure adapted from Fig. 8A - B of Albert *et al.*, 2011.
https://doi.org/10.1111/j.1365-313X.2010.04465.x

Figure 1.5: Regulation of anthocyanin pigmentation in *Petunia* (figure adapted from Albert et al., 2011). Four closely related transcription factors are responsible for the visible pigmentation pattern in *Petunia*. *AN2* controls visible color in the petal limb while *AN4* controls pigmentation in the flower tube and anthers. *PHZ* is responsible for flower bud blushing and pedicel pigmentation while *DPL* controls coloration in the flower tube veins.

Not only do R2R3-MYB transcription factors activate the production of visible color pigmentation in *Petunia*, but also UV color. UV absorption in flowers has long been attributed to flavonols (Birkofer and Kaiser, 1962; Wiering, 1974; Griesbach and Asen, 1990). *MYB-FL*, a related R2R3-MYB transcription factor, is responsible for flavonol accumulation in the floral limb in *Petunia* (Sheehan et al., 2016). Plants with a functional *MYB-FL* gene produce flowers that appear UV absorbent due to the accumulation of flavonols, while plants without a functional *MYB-FL* appear UV reflective (Sheehan et al., 2016). Contrary to the transcription factors controlling visible color, *MYB-FL* homologs in *Arabidopsis* have been shown to not require a bHLH factor to activate targets (Zimmermann et al., 2004; Mehrtens et al., 2005). These visible and UV color pigments do not only affect floral pigmentation individually, but can interact together as co-pigments. This can lead to complex inter-molecular interactions that may alter the visual hue of flowers. As the *AN2* and *MYB-FL* genes control pigmentation of the petal limb, they together control the floral color display that is presented to pollinators.

Speciation genes controlling color in *Petunia*

Given the importance of the two transcription factors *AN2* and *MYB-FL* in controlling visible and UV floral color in *Petunia*, mutations that change protein functionality and the arising phenotypes can heavily influence pollinator preference and choice (Esfeld et al., 2018; Hoballah et al., 2007; Sheehan et al., 2016). The specific major phenotypic effects

controlled by these genes confer changes in pollinator choice, and thus affect reproductive isolation, elevating them to be considered as "speciation genes" (Hoballah et al., 2007; Sheehan et al., 2016). Transitions between pollination syndromes in wild *Petunia* species have been attributed to changes in these color speciation genes (Table 1.1). In the long tube clade *P. axillaris*, which absorbs UV light, has a functional *MYB-FL* gene, which confers high levels of flavonols. Conversely, UV-reflective *P. exserta* contains a 1 bp deletion in the *MYB-FL* gene, resulting in a truncated protein (Sheehan et al., 2016). Hawkmoths significantly preferred to feed from UV-absorbent *P. axillaris* flowers when presented with flowers differing in UV color but not visible color, demonstrating the strong influence of this single gene on pollinator preference (Sheehan et al., 2016).

A similar pattern can be observed for the visible color speciation gene: in the short tube species *P. inflata,* visible color is produced through a functional *AN2* gene, that activates the production of anthocyanins in the flavonoid biosynthetic pathway (Esfeld et al., 2018). In *P. axillaris*, loss of visible color was attributed to at least six different *AN2* haplotypes, all of which encode nonfunctional proteins (Esfeld et al., 2018). When given the choice between *P. axillaris* and *P. inflata*, bees significantly preferred the colored species *P. inflata* with a functional *AN2* gene (Hoballah et al., 2007). Hawkmoths displayed opposite preferences and preferably visited *P. axillaris* plants (Hoballah et al., 2007). However, an important limitation in this comparison is that the phenotypic differences between the species are not due to a single gene. This was solved by presenting bee and hawkmoth pollinators with transgenic *P. axillaris* lines where the non-functional *AN2* copy was complemented with a functional copy (Hoballah et al., 2007). Bees significantly preferred the transgenic line with a functional *AN2* while hawkmoths preferred the white colored wildtype (Hoballah et al., 2007). These comparisons underline the importance of this transcription factor in reproductive isolation and why it is considered to be a speciation gene.

While the white colored *P. axillaris* species of the long tube clade have a non-functional *AN2* gene, *P. secreta* with its purple-magenta color, is the only species in this clade that has regained *AN2* function through an additional 2 bp deletion, restoring the reading frame of the protein (Esfeld et al., 2018). This single reading-frame-restoring mutation demonstrates the regain of floral color during *Petunia* evolution and speciation, a

remarkably simple evolutionary transition (Esfeld et al., 2018). Field studies have demonstrated that *P. secreta* is pollinated by solitary bees (Rodrigues et al., 2018a) but precise pollinator choice assays comparing *P. secreta* to other *Petunia* species are still lacking and much needed in order to determine the relative importance of this speciation gene in all transitions within the *Petunia* clades.

The evolutionary history of *Petunia* speciation across the short and long tube clades suggests the transition from bee to hawkmoth and either back to bee or to hummingbird pollination syndrome (see Fig. 1.2). The activation of *MYB-FL* and inactivation of *AN2* allowed the shift from bee to hawkmoth pollination to occur, while the inactivation of *MYB-FL* was required to obtain bee and hummingbird pollination in the long tube clade. Regaining function of *AN2* was presumably needed to transition back to bee syndrome in *P. secreta*, but this has yet to be validated through behavioral assays with this species. Color regain in the hummingbird syndrome was through a more complicated mechanism involving multiple genetic alterations (Berardi et al., 2021). Overall it seems that few changes in major effect genes responsible for visible color differences (Table 1.1) account for the majority of the transitions between pollination syndromes.

Floral scent as a signal for pollinators

Not only are visual cues of importance to help attract and guide pollinators, but olfactory cues as well (Raguso, 2001; Reinhard et al., 2004). The specific attraction of certain pollinators through scent volatiles has been demonstrated for multiple pollinator species (Huber et al., 2005; Klahre et al., 2011), but moths in particular are highly sensitive to flower odors (Raguso et al., 1996; Raguso and Willis, 2002). They are also capable of rapidly learning flower odors (Daly and Smith, 2000) and can spatially discriminate where odor plumes are located (Parthasarathy and Willis, 2021). This allows for highly discriminating pollinators, which can lead to reproductive isolation of species through specific selection of plants based on their phenotypes. Scent compounds attracting pollinators are therefore highly important in the *Petunia* system.

Numerous volatile compounds are emitted by different fragrant *Petunia* species and are produced through the benzenoid-phenylpropanoid biosynthetic pathway (as shown in

Fig. 1.6, adapted from Amrad et al., 2016). The transcription factor *ODO1* is responsible for activation of the shikimate pathway that leads to the production of the precursor phenylalanine. The precursor phenylalanine is modified by pathway branching enzymes PAL and PAAS. PAL produces the subsequent precursor t-Cinnamic acid that is the target for the enzyme CNL, which through a further series of modifications eventually produces the benzenoids. These compounds are of major importance for attracting pollinators as three compounds elicited the highest responses when presented to hawkmoths (Hoballah et al., 2005). The precursor *t*-Cinnamic acid can also be modified by the enzyme C4H and other biosynthetic enzymes to produce the phenylpropanoids. Although the phenylpropanoids are major odor components of some *P. axillaris* accessions, these compounds did not elicit as strong of a response as benzenoid compounds when presented to hawkmoths (Hoballah et al., 2005). Specifically attracting pollinators through odor cues therefore needs to occur through a combination of the correct volatile compounds and their ratios.

Figure adapted from Fig. 1 of Amrad *et al.*, 2016.
http://dx.doi.org/10.1016/j.cub.2016.10.023

Figure 1.6: Benzenoid-phenylpropanoid biosynthetic pathway in *Petunia* (adapted from Amrad et al., 2016). The transcription factor *ODO1* activates the shikimate pathway to produce the precursor phenylalanine. Both benzenoid and phenylpropanoid compounds are produced from this precursor. Transcription factors are shown in purple and enzymes in blue. For enzyme names refer to abbreviations. Dashed arrows depict enzymes and genes that have not yet been described in *Petunia*. Figure was created using Biorender.com.

Not only is the composition of scent compounds important for pollinator attraction, but also the timing of their emission. Scent genes are expressed in a circadian manner, which is crucial for pollinator attraction during a certain time period during the day or night (Hoballah et al., 2005; Amrad et al., 2016). The morning expressed clock component PhLHY in *P. hybrida* directly influences the expression of genes related to floral scent.

During the daytime PhLHY represses the expression of genes related to floral scent, thus restricting the emission of scent to nighttime (Fenske et al., 2015). The synchronization of the circadian clock of *Petunia* to release floral scent compounds and pollinator flight is important for moth visitation preference (Hoballah et al., 2005; Fenske et al., 2018). If the plants are desynchronized to the daily rhythm of the moths or the moths are desynchronized to the circadian rhythm of the plants, pollinator recognition is not as efficient (Fenske et al., 2018). Both the plant and pollinator's circadian clocks need to be synchronized to initiate their interaction (Hoballah et al., 2005; Fenske et al., 2018).

Scent emission speciation genes in *Petunia*

As scent emission is an important factor affecting pollinator attraction, specific differences in odor emission between the different *Petunia* species have been documented. *P. axillaris*, pollinated by hawkmoths, emits multiple volatiles belonging to the floral benzenoids and phenylpropanoids while *P. inflata*, pollinated by bees, only emits benzaldehyde (Hoballah et al., 2005). On the other hand, *P. exserta*, pollinated by hummingbirds, does not produce any scent volatiles (Amrad et al., 2016). There have been multiple major effect scent genes identified in *Petunia* (Table 1.1): R2R3-MYB transcription factor *ODO1* and biosynthetic genes *CNL1, BSMT and BPBT* (Klahre et al., 2011; Amrad et al., 2016). High levels of *ODO1* can be found in *P. axillaris*, correlating with high amounts of scent emission (Klahre et al., 2011). *P. exserta* on the other hand produces lower amounts of *ODO1* transcripts, most likely due to differences in promoter activity (Klahre et al., 2011). Including a *P. axillaris* copy of *ODO1* in introgression lines demonstrated that this was sufficient to elevate levels of *ODO1* and induce higher scent volatile production. In *P. axillaris* the *CNL1* gene is intact, but is inactivated through multiple mutations in *P. exserta* (Amrad et al., 2016). Introducing the functional *CNL1* gene of *P. axillaris* into a plant background producing no scent significantly increased methylbenzoate production, while introducing the disabled *CNL1* gene of *P. exserta* could not restore scent production (Amrad et al., 2016). This demonstrates the causal effect of *CNL1* on the reduction of benzenoid synthesis in wild *Petunia* species (Amrad et al., 2016). The different bouquet of scent volatiles produced in *P. inflata* is not due to any differences in the coding region of *CNL1*, but because of expression differences in *CNL1* as well as in genes *BSMT* and *BPBT* encoding enzymes further downstream (Amrad et al., 2016;

Bombarely et al., 2016). These differences between species demonstrate the importance of *ODO1*, *CNL1, BSMT and BPBT* in transitions during *Petunia* speciation.

The transitions during *Petunia* speciation from bee to hawkmoth to either hummingbird or bee pollination involved an increase in complexity and concentration of scent volatiles for bee to hawkmoth pollination (Bombarely et al., 2016) and a subsequent elimination of volatile emission from hawkmoth to hummingbird pollination (Amrad et al., 2016). The shift from bee to hawkmoth pollination must have involved mutations in *cis*-acting regulatory elements as proteins of all known scent biosynthetic and regulatory genes are predicted to be functional (Amrad et al., 2016; Bombarely et al., 2016). Additionally the upregulation of structural genes in *P. axillaris* seems to increase the overall amount of volatiles (Amrad et al., 2016). The subsequent shift from hawkmoth to hummingbird pollination included the functional inactivation of a structural gene as well as a transcriptional regulator to account for the loss of scent in *P. exserta* (Amrad et al., 2016; Klahre et al., 2011). While the role of scent volatiles in the transition from hawkmoth to hummingbird syndrome has been studied in detail, the loss of scent in the transition from hawkmoth to bee syndrome in *P. secreta* is not as well yet understood. Preliminary data shows that scent reduction in *P. secreta* is most likely caused by a reduction in expression of both *ODO1* and *CNL1* (A. Berardi, unpublished data). In all transitions involving scent gain or loss in *Petunia*, the same major effect genes seem to account for a majority of the phenotypic variation (Table 1.1).

Table 1.1. Identified major effect genes in *Petunia* contributing to shifts in pollination syndromes between species. NA depicts unknown values in genes that have not yet been studied in detail in the respective species.

Gene	*P. inflata*	*P. axillaris*	*P. exserta*	*P. secreta*
	bee	***hawkmoth***	***hummingbird***	***bee***
AN2	active	inactive	inactive	active
MYB-FL	strongly reduced	active	inactive	inactive
ODO1	active	active	strongly reduced	strongly reduced
CNL1	active	active	inactive	strongly reduced
BSMT	strongly reduced	active	active	NA
BPBT	strongly reduced	active	NA	NA

Detection of floral signals by pollinators

When studying various pollination syndromes, it is essential to use a combination of pollinators that have been well established as model systems for lab experiments but also reflect the pollinators that occur in the natural habitat range as much as possible. Two well-established pollinator systems are those of *Bombus terrestris* (bumblebees) and *Manduca sexta* (hawkmoths). *B. terrestris* is a polylectic bee that collects from a wide range of different host species (Rasmont, 1988) making it possible to test them in experiments with various plant species. Its distribution ranges from Europe, Africa, New Zealand to South America (Macfarlane and Gurr, 1995; Winter et al., 2006). *B. terrestris* are eusocial insects and form colonies with reproductive division of labor and an annual life cycle, where only the queens overwinter to form new colonies in spring. Worker bees collect nectar and pollen to bring back to the nest for the larvae, making them an ideal pollinator to study foraging strategies for rewarding traits. Their foraging range and frequency depends on the availability of host plants in the area, but can reach over a kilometer in distance (Osborne et al., 2008).

The hawkmoth, *M. sexta*, is native to North and South America. The larvae feed on a variety of mostly solanaceous plants while the adults are nocturnal and feed on the nectar of flowers. *M. sexta* hawkmoths can migrate for tens of kilometers and unlike bumblebees

have no specific home range (Janzen, 1984; Haber and Frankie, 1989; Pittaway, 1993). They can easily be reared in the lab over many generations on an artificial diet, making them an excellent laboratory pollinator system. This animal has been used extensively in neurobiological and scent research (Willis and Arbas, 1991; Homberg et al., 1988; Riffell et al., 2009; Goyret and Yuan, 2015) and has also become a model for research in plant-insect interactions (Orozco-Cardenas et al., 1993; Goyret et al., 2008; Reisenman et al., 2013; Haverkamp et al., 2016a; Garvey et al., 2020).

In order to find specific flowers, insects must rely on their vision or scent, or both. Insect vision differs from human vision, which is why consideration of the insect pollinator visibility spectrum and how it overlaps with the spectrum of floral pigments is crucial. Both bumblebees and hawkmoths have a trichromatic visibility spectrum (Schwemer and Paulsen, 1973; Bennett and Brown, 1985; Peitsch et al., 1992; Kelber et al., 2003; Dyer et al., 2011a) spanning the UV (300-400 nm), blue (400-500 nm) and green (500-600 nm) color ranges (Fig. 1.7, adapted from Kelber et al., 2003 and Dyer et al., 2011a). Bumblebees are only able to resolve relatively low spatial frequency information due to their compound eyes (Spaethe and Chittka, 2003) and as bees are mainly active during the day, they are attracted by the visible color of flowers such as pink, purple and yellow (Chittka and Raine, 2006). Depending on the reflectance that occurs in visible color ranges of a bee's visibility spectrum, UV reflectance can increase or decrease the detectability of a flower (Kevan et al., 2001). For example for white colored flowers, UV reflectance can significantly prolong a bee's search time (Spaethe et al., 2001) while for red colored flowers bees prefer UV reflecting flowers over UV absorbing ones (Chen et al., 2020). It seems that color contrast between the target and background is more important for bees to detect a flower than the sole presence or absence of UV reflectance (Giurfa et al., 1996; Kevan et al., 2001).

Hawkmoths also use color vision for floral detection/visitation and possess color constancy, that is, the ability to recognize specific colors even under changing illumination (Kelber et al., 2003). However, nocturnal hawkmoths are active around dusk or at night, and they thus often rely on UV color to guide them and must have highly light sensitive eyes (Kelber et al., 2003). Given that the foraging behaviors of diurnal and nocturnal pollinators occur under very different lighting conditions, nocturnal

pollinators have developed specific adaptations to cope with these low light conditions. Even under low light conditions, chromatic cues (wavelength-related) are used as opposed to achromatic cues (intensity-related) (Kelber et al., 2003). Hawkmoths are able to adjust their vision to dim light by slowing down their visual processing (Sponberg et al., 2015) and neural summation (Stöckl et al., 2016). Under different lighting conditions, hawkmoths are also able to trade spatial resolution for higher sensitivity (Stöckl et al., 2020). Using visual cues is also important for their flight control during hovering (Farina et al., 1994, 1995), as hawkmoths do not land on a flower when foraging. These low light conditions are also why *M. sexta* moths depend on both visual and olfactory cues for flower detection and feeding (Raguso and Willis, 2002).

Figure adapted from Fig. 1A of Kelber *et al.*, 2003 and Fig. 1 of Dyer *et al.*, 2011a.
https://doi.org/10.1093/icb/43.4.571
https://doi.org/10.1098/rspb.2010.2412

Figure 1.7: Trichromatic visibility spectrum of bees and hawkmoths. The visibility spectrum of both hawkmoths and bees expands into the UV, blue and green range (adapted from Kelber et al., 2003 and Dyer et al., 2011a).

Further factors influencing the detection of floral signals by pollinators

In addition to the influence of floral pigments on the appearance of a flower, the shape of petal epidermal cells influences how light enters the pigment containing cells and thus how these colors appear (Dyer et al., 2007). *Antirrhinum* flowers with a mutation in the

MIXTA gene, a *MYB* transcription factor that controls petal epidermal cell shape, appeared pink instead of the wildtype purple flowers, and did not appear as shiny to the human eye (Dyer et al., 2007). This shift in hue was due to the conical shaped epidermal cells that were flat in the mutant, and reduced flower color saturation by altering the proportion of light able to enter the vacuoles containing pigments (Dyer et al., 2007). From a human perspective, one could conclude that these differences should make the *mixta* mutants less attractive to pollinators. However, the wildtype and mutant flowers actually displayed very similar color contrast for bees and no significant differences in accuracy or speed could be observed when bees detected the flowers (Dyer et al., 2007). Pollinators could in fact learn to discriminate between wildtype and *mixta* flowers if trained to do so but the flowers were so similar in color that they made many errors. Larger color distances are easier for bumblebees to discriminate than smaller color differences (Dyer and Chittka, 2004a; Dyer et al., 2007) and bumblebees require training with target and distractor colors (differential conditioning) to successfully complete fine color discrimination tasks (Dyer and Chittka, 2004b). The results observed for bumblebees were not intuitive when inspecting the flowers using human vision, underlining the necessity to consider pollinator specific perception.

An additional factor impacting pollinator attraction is the patterning of floral pigmentation. Different UV absorbing or reflecting patterns can help guide pollinators to the floral reward (Gronquist et al., 2001; Koski and Ashman, 2014; Brock et al., 2016). UV patterns are usually associated with bee pollinated flowers and are referred to as nectar guides. They have been proposed to act either as long-distance cues for attracting bee visitors (Horth et al., 2014; Koski and Ashman, 2014; Peterson et al., 2015) or help the bee orient itself and find the nectar once it has reached the flower (Eugene Jones and Buchmann, 1974; Papiorek et al., 2016). *Mimulus guttatus* flowers for example display a nectar guide acting as a "runway" for bees. Their lower petal is UV absorbent, helping to attract and orient wild bee pollinators (Rae and Vamosi, 2013). Other flowers, such as *Rudbeckia hirta, Argentina anserina* or *Hemerocalli citrina*, show a typical "bull's eye" pattern, with a UV absorbing center and UV reflecting peripheries (Schlangen et al., 2009; Koski and Ashman, 2014; Hirota et al., 2019). This bull's eye pattern has not only been shown to attract bee pollinators (Koski and Ashman, 2014) but has also been shown to have a direct effect on hawkmoth pollination, with the UV pattern affecting the

hawkmoth's choice more than visible color differences (Hirota et al., 2019). In *Petunia*, the species in the long tube clade do not display any UV patterning, while *P. inflata* in the short tube clade shows a "bull's eye" pattern (Sheehan et al., 2016). This UV patterning in the short tube clade may make *P. inflata* more attractive to bee pollinators than the bee pollinated species *P. secreta* in the long tube clade. Bee pollination success could therefore be higher in the short tube clade through the presence of such nectar guides.

Specific adaptations of a pollinator to its host promote successful pollination

Pollination success depends on how well a pollinator is able to detect a flower but also how quickly it is able to do so. To detect floral signals as quickly as possible when they start foraging, pollinators have innate preferences for floral color and scent (Giurfa et al., 1995; Kelber, 2002; Riffell et al., 2008). They are also capable of learning to associate floral signals with rewards during their lifespan (Weiss and Papaj, 2003; Balkenius and Kelber, 2006). Multiple pollinator species can rapidly learn to associate olfactory or visual cues with specific rewards. Learning occurs quicker if pollinators are required to learn to associate a reward with a color they innately prefer as opposed to other colors. Bumblebees have two systems for processing color information in their brain: a rapid discrimination pathway that is capable of making decisions about rewarding or non-rewarding flowers and a slow learning-dependent pathway used for long-term decisions such as discriminating between flowers and mimics that are perceptually similar (Dyer et al., 2011b). Such acquired preferences are not restricted to only one cue type, but also play a role in the interaction of olfactory and visual cues. The odor learning of hawkmoths for example was manipulated by using different colors (Balkenius and Kelber, 2006) demonstrating how plastic this system is.

Once a flower has been detected as quickly and efficiently as possible, pollination success for the plant can vary greatly depending on the type of pollinator that visits. Pollinators can be divided into two large groups depending on what host plants they visit: generalist and specialist pollinators. Specialist pollinators are usually well-adapted to their narrow range of host plants and are therefore more efficient in pollinating a plant. Specialized bees, for example, have adaptations to flowers that enhance their pollen collecting efficiency in host plants. These adaptations can be of a morphological or behavioral

nature and make them strong competitors and more effective pollinators compared to generalist bees (Alves Dos Santos and Wittmann, 2000; Schlindwein and Martins, 2000; Alves Dos Santos, 2002; Carvalho and Schlindwein, 2011; Cerceau et al., 2019).

In hawkmoths, morphological and behavioral adaptations can also be observed. Their proboscis length is often adapted to their host plant allowing only certain moth species to access the reward offered by the plant. Certain hawkmoth-plant pairs have adapted behavioral strategies where the female hawkmoths use the plant both for nectar foraging (Alarcon et al., 2008; Riffell et al., 2008) and oviposition (Mechaber and Hildebrand, 2000). Hawkmoths have also adopted cost efficient foraging strategies (Haverkamp et al., 2016b). Their foraging strategies are energetically demanding as they do not land on the flower but feed while hovering, which consumes energy. Adapting their foraging strategy to flowers matching the length of their proboscis allows them to get the highest net-energy reward (Haverkamp et al., 2016b). The same can be observed for other animal pollinators, such as hummingbirds, that have high energy consuming strategies. As nectar in a flower is often concealed from the pollinators, hawkmoths must evaluate the nectar content through insertion of their proboscis. They are therefore also capable of adapting their probing time depending on whether a flower offers a large nectar reward or not (Brandenburg et al., 2012a, 2012b). Hawkmoths reduced their probing time in both low-nectar bred lines and manually reward-minimized lines (Brandenburg et al., 2012a, 2012b). This reduction in probing time also negatively affected the plant's reproductive success as a reduced seed set was found in the plants with low nectar availability (Brandenburg et al., 2012b). From the plant's perspective, attracting the ideal pollinator that is adapted for pollination through various mechanisms, is therefore vital for successful plant-insect interactions.

Reconstitution of pollinator-mediated speciation

The question to what extent few genes of large effect contribute to evolutionary transitions has been a long-standing debate. As we are forced to analyze the process of speciation in hindsight, retracing the evolutionary steps of divergence is crucial to answering this question. This process is genetically virtually intractable with current methods in species that have diverged exclusively through an infinite number of small effect mutations. However, mutations of large effect are now tractable and have been

identified in a wide range of systems. One such system is *Petunia* in which multiple speciation genes have been described to date (Quattrocchio et al., 1999; Hoballah et al., 2007; Amrad et al., 2016; Sheehan et al., 2016). Testing the effect of mutations in speciation genes at the level of reproductive isolation (the pollinators) now becomes possible.

The project presented in this thesis aimed at targeting previously identified speciation genes through a CRISPR/Cas9 approach to precisely modify single genes and observe their effect on pollinator preference. The CRISPR/Cas9 system offers a unique opportunity to target a single speciation gene and demonstrate the effect of a precise molecular mutation on both the plant phenotype and the behavioral response of pollinators. Outcrossing of the Cas9 cassette in subsequent generations eliminates off-target effects based on the insertion position of this sequence. The **first aim** of this project was to establish a stable CRISPR/Cas9 transformation protocol for wild *Petunia* species (*P. axillaris* lines, *P. secreta*) to successfully alter their genome. This included developing the gene constructs for transformation and the conditions being used for various transformation processes.

P. secreta represents a reversal from hawkmoth to bee pollination. This involved loss of flavonols and regain of anthocyanins due to loss of *MYB-FL* function and regain of *AN2* function, respectively (Esfeld et al., 2018). To recreate this evolutionary transition in the lab, the **second aim** was to render the *MYB-FL* gene in *P. axillaris* non-functional while also restoring function of the *AN2* gene. The **third aim** was to then test the evolutionary impact mutations in these single genes have, through pollinator behavioral assays. Using reciprocal comparisons with the respective parental lines, as well as different pollinator types that are associated with different pollination syndromes, the precise difference a single gene makes on the preference of pollinators and ultimately the events that happen through pollinator-mediated speciation can be determined.

The specific aims of this study were:

- To target the *AN2* gene responsible for visible color pigmentation in *Petunia* and restore color in the hawkmoth pollinated *P. axillaris* plant through CRISPR/Cas9

as well as other transgenic approaches, thus creating a shift in floral color (Chapter 2).

- To target the *MYB-FL* gene responsible for UV color pigmentation in *Petunia* and alter the UV absorption to mutate a species with UV absorbent flowers and create UV reflective flowers (Chapter 3).
- To perform pollinator assays on these UV-reflective mutants and determine behavioral differences in pollinators through a single speciation gene that may lead to reproductive isolation (Chapter 3).
- To test multiple different pollinator species (hawkmoths and bees) to see how changes in speciation genes affect their foraging behaviors (Chapter 3).
- To combine multiple mutated speciation genes in a single *P. axillaris* plant and thereby mimic the transition from hawkmoth to bee pollination syndrome (Chapter 4).

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Chapter 2

Partial visible color restoration through the *AN2* gene in a hawkmoth pollinated background in *Petunia*

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Author contributions

Martina Lüthi: design of constructs for CRISPR/Cas9 gene editing and *AN2* expression with *CaMV35S*, native and *CHS* promoters; genetic manipulation of wild *Petunia* species; DNA extractions and analyses of *AN2* sequences; UV/VIS photography and spectrophotometric measurements and analyses; RNA extractions for RT-qPCR experiments; RT-qPCR experiments and analyses; graphical visualization of all analyzed data; statistical analyses; writing of manuscript

Andrea E. Berardi: spectrophotometric analyses; reviewing of manuscript

Cris Kuhlemeier: conceptualization; supervision

Abstract

Shifts in pollinators often involve changes in visible color caused by floral pigmentation differences produced through the flavonoid biosynthetic pathway. The genus *Petunia* displays discrete pollination syndromes (floral traits associated with the attraction of specific pollinators) allowing transitions in visible colors and associated pollinator shifts to be studied. The R2R3-MYB transcription factor *AN2* controls anthocyanin biosynthesis in the floral limb in *Petunia*. In the white colored species *P. axillaris*, *AN2* function has been disabled through inactivating mutations while the purple colored species *P. secreta* has regained visible color through a restoration of the *AN2* gene. Here we aimed to restore *AN2* function in a colorless background (*P. axillaris*) through multiple gene editing and transgenic complementation approaches. The *CHS-A* promoter, a strong petal-specific promoter, induced the strongest change in visible color, through expression of a functional *AN2* gene. However, full color to the extent observed in *P. secreta* was not detected, as anthocyanidin absorbance was elevated in the transgenic plants but not as high as in *P. secreta*. Introducing a functional *AN2* gene also induced changes in genes responsible for color production, demonstrating a downregulation of genes responsible for flavonol production (*MYB-FL* and *FLS*) and an upregulation of genes involved in anthocyanin production (*ANS* and *DFR*). Not all genes upregulated in *P. secreta* relative to *P. axillaris* showed a higher expression in the *AN2* transgenics, underlining the necessity for further genes being required to restore complete visible color.

Introduction

As plants are sessile they are dependent on vectors to effectively disperse pollen or seeds. Depending on the geographical region, between 78% to 94% of flowering plants are estimated to be pollinated by animal vectors (Ollerton et al., 2011). This mutually beneficial relationship between plants and their animal pollinators is critical to the reproductive success of the majority of plant species. Pollinators provide pollination services to plants through pollen transportation, facilitating the exchange of genetic material between different plants and populations. In return pollinators gather rewards from the plants, such as nectar and/or pollen. They are able to use different floral traits and signals to locate floral rewards and discriminate between different plant species (Chittka and Raine, 2006). Plants therefore try to advertise their rewards as best possible, ideally attracting the most efficient pollinator to maximize pollination success.

One of the main mechanisms for a plant to advertise its rewards to potential pollinators is through floral color. Floral color has been shown to be a major determinant for attracting pollinators (Hoballah et al., 2007; Campbell et al., 2010). Pollinators often strongly rely on color for making their foraging decisions (Heiling et al., 2003; Ômura and Honda, 2005). Innate preferences for certain colors have been shown for multiple pollinator species (Lunau and Maier, 1995; Raine and Chittka, 2007; Kuenzinger et al., 2019) demonstrating the importance of a well-adapted plant-pollinator interaction.

Shifts in pollinators often involve changes in visible color (Bradshaw and Schemske, 2003; Hoballah et al., 2007; Esfeld et al., 2018; Berardi et al., 2021). Loss of visible flower color is predominantly due to mutations in regulatory genes (Quattrocchio et al., 1999; Durbin, 2003; Schwinn et al., 2006; Hoballah et al., 2007; Streisfeld and Rausher, 2009a; Yuan et al., 2013) while color transitions are often caused by mutations in biosynthetic genes (Zufall and Rausher, 2004; Streisfeld and Rausher, 2009b; Des Marais and Rausher, 2010; Hopkins and Rausher, 2011; Smith and Rausher, 2011; Wessinger and Rausher, 2012, 2014). Different mechanisms can lead to either inactivation, deletion or downregulation of these pathway genes. Understanding the mechanisms underlying these transitions is important for analyzing the evolutionary mechanisms of floral color change.

It is therefore not surprising that color is one of the main traits in pollination syndrome theory (Faegri and Pijl, 1979). Pollination syndromes describe a set of traits associated with attracting a specific type of pollinator. Convergent evolution of floral traits should thus be seen in unrelated plant species adapted to the same pollinator (Faegri and Pijl, 1979). The pollination syndrome concept has classified such pollinators into functional groups that behave similarly leading to similar selection pressures being exserted on floral displays of plants (Fenster et al., 2004). Multiple examples of plant species with converging floral traits exhibiting the same pollination syndromes have been found to validate pollination syndrome theory (Rosas-Guerrero et al., 2014; Smith and Kriebel, 2018; Lagomarsino and Muchhala, 2019).

The South American genus *Petunia* offers a compelling system where transitions in visible colors and associated pollinator shifts can be studied. This genus comprises species with different pollination syndromes associated with varying floral colors to attract different pollinator species. Two major clades have been identified in the *Petunia* genus: the short tube and long tube clade (Reck-Kortmann et al., 2014). The short tube clade encompasses the majority of the species, which are all bee pollinated. To attract these bee pollinators, the short tube clade species all have very similar phenotypes with purple colored flowers, exhibiting the same bee pollination syndrome (Reck-Kortmann et al., 2014). In the long tube clade, however, many key color changes have been demonstrated, including losses, gains and shifts in color (Hoballah et al., 2007; Esfeld et al., 2018; Berardi et al., 2021). This clade encompasses species ranging from white, red to purple in color (Esfeld et al., 2018) corresponding to hawkmoth, hummingbird and bee pollination syndromes respectively (Reck-Kortmann et al., 2014).

Differences in floral pigmentation are produced through flavonoids in a majority of the angiosperms. The flavonoid biosynthetic pathway produces both visible (anthocyanins) and UV (flavonols) color pigments from common precursors and both pigment types can be found in *Petunia* petals (Sheehan et al., 2016; Esfeld et al., 2018). Transitions in visible colors of *Petunia* species have been attributed to changes in genes of major effect in the flavonoid biosynthetic pathway. As many transitions between pollination syndromes can be found in the long tube clade of *Petunia*, such mechanisms have been described in detail for these species. The most recent phylogeny robustly separates the short tube and long

tube clade while placing *P. axillaris* as sister to the two colored species *P. exserta* and *P. secreta* in the long tube clade (Esfeld et al., 2018) suggesting that the gain of visible color occurred from a colorless ancestor in both species. Anthocyanin biosynthesis in the *Petunia* floral limb is specifically controlled through the R2R3-MYB transcription factor *AN2* and its phylogenetic history supports the notion that the colored species have evolved from a colorless ancestor (Esfeld et al., 2018). Inactivating mutations in the *AN2* gene have led to a loss of visible color in the white-flowered species *P. axillaris* (Quattrocchio et al., 1999; Hoballah et al., 2007). As breaking something is often easier than repairing it, the regain of red color in *P. exserta* has occurred through a more complicated mechanism where multiple genetic alterations are required (Berardi et al., 2021). Contrary to this, the regain of color in the purple colored species *Petunia secreta* has been surprisingly simple: an additional 2 bp deletion in the *AN2* gene has restored the reading frame and allowed *P. secreta* to produce anthocyanin pigments (Esfeld et al., 2018).

It is this seemingly easy solution to a difficult problem that led us to investigate whether the evolutionary transition from hawkmoth to bee pollination syndrome can be recreated in the laboratory. As the *AN2* gene has been shown to be a major effect gene leading to shifts in pollinator visitation, recreating the mutational steps that led to this shift in visible color would demonstrate how this single gene alteration affects pollinator behavior, ultimately influencing reproductive isolation. Loss-of-function experiments via a VIGS approach targeting *AN2* were able to demonstrate that *P. secreta* loses its visible color if *AN2* is silenced (Esfeld et al., 2018). If the regain of *AN2* function is as simple as has been suggested, restoring *AN2* function in a white-colored flower should lead to a gain of visible color. However, gain-of-function experiments using the *CaMV35S* promoter to express the *AN2* gene of *P. secreta* in a *P. axillaris* background displayed phenotypic differences between *P. secreta* and the *AN2* transgenics as full visible color was not restored (Esfeld et al., 2018). The same light pink phenotype was observed in experiments where transgenic complementation was performed with an identical promoter but using the *AN2* gene from the short tubed species *P. integrifolia* (Hoballah et al., 2007). Partial color restoration therefore seems to be independent of the *AN2* gene being used for complementation, but may depend on the promoter controlling the expression of the gene.

Following up on these findings will therefore allow us to answer important questions regarding the restoration of color in *P. secreta* and the shift from hawkmoth to bee pollination syndrome in the long tube clade. What will the phenotypic effects of this alteration in a single gene be? Will visible color be restored completely through a mutation in *AN2* or is there residual variation in visible color? Can we restore visible color to its full extent through an overexpression approach of the *AN2* gene using different promoters? If we cannot restore full color what factors could be contributing to the residual variation we can observe?

Using a CRISPR/Cas9 approach we aimed to target the *AN2* gene in *P. axillaris* and recreate the 2 bp deletion that can be found in *P. secreta*. To analyze the regain of color from white to purple through multiple approaches, we also set up an overexpression approach for the *P. secreta AN2* gene in floral limb tissue using different promoters. Analyzing various approaches should allow us to assess if the degree of color restoration is dependent on the approach used. Mimicking the hawkmoth to bee transition in *Petunia* species through either one of these approaches should lead to a shift in floral color from white to purple by targeting a single gene.

Materials and methods

Plant material

P. axillaris ssp. axillaris N (hereafter referred to as *P. axillaris* N) originates from the Rostock Botanical Garden in Germany and is registered in the Amsterdam collection as P. axillaris S26. *P. axillaris ssp. axillaris* P (hereafter referred to as *P. axillaris* P) originates from the University of Bern Botanical Garden while *P. axillaris* O originates from the Technical University Dresden Botanical Garden (Hoballah et al., 2007). *P. secreta* was collected in its natural habitat Galpão de Pedra, Rio Grande do Sul, Brazil. All accessions were maintained by selfing. Plants were grown in commercial soil (70% Klasman substrate, 15% Seramis clay granules, 15% quartz sand) under a light:dark regime of 15:9 h at 22:17 °C in a growth chamber. Plants were fertilized once a week with a nitrogen-phosphorous-potassium and iron fertilizer.

Color image scoring

Color images were recorded with a Canon EOS 60D camera and Canon 35 mm lens (aperture F4.5 – F7.0, ISO 100 – 200, shutter speed 1/30 s). All images were captured under identical incandescent lighting and with a black background for standardized comparison with a color calibration card (X-Rite ColorChecker Classic Mini).

Spectrophotometric quantifications of floral flavonols and anthocyanidins

Total flavonol and anthocyanidin (anthocyanin aglycone) absorbance was measured for all flower petal limb samples using an Ultraspec 3100 pro (GE Healthcare Life Sciences) spectrophotometer, at 365 nm and 530 nm. Three discs (8 mm diameter) were punched out of the floral limb tissue and placed in 1 ml of 2N HCl to soak for 15 min at room temperature (22 °C), hydrolyzed at 100 °C for 15 min, cooled for 10 min at 4 °C, then centrifuged for 3 min at 14000 rpm. Supernatants were analyzed directly to avoid degradation of pigments. Absorbance for each sample was measured at 530 nm for anthocyanidins (Harborne, 1998) and 365 nm for flavonols (Harborne, 1998). Five flowers were sampled per plant to include technical replicates for each biological replicate (individual plant). Statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463 as data was normally distributed. Data was visualized using the ggplot2 package (Wickham, 2016).

Generating *P. secreta AN2* overexpression constructs

Six constructs with different promoters were designed to express the *P. secreta AN2* gene in *P. axillaris*. The promoters used for the constructs were: *CaMV35S* promoter as in (Albert et al., 2011), native *AN2* promoter of *P. secreta* (3 kb length), native *AN2* promoter of *P. inflata* (3 kb length), *CHS-A* promoter (3 kb length), *CHS-J* promoter (3 kb length) and *CHS-A* promoter (800 bp length). These promoter fragments were used in constructs upstream of the *P. secreta AN2* gene. Downstream of the *AN2* gene, 0.5 kb of the downstream sequence (3' UTR) were also included to account for possible enhancing factors. L sites for Gateway reactions were added up- and downstream of the promoter and gene sequences. The entire fragments for cloning (promoter + *AN2* gene + 3' UTR site + L cloning sites) were synthesized by Genscript Biotech Corporation (complete sequences of gene fragments in Suppl. Table S2.5). The constructs were then cloned into

pGWB401 destination vectors using the LR reaction from the Gateway cloning protocol (Thermo Fisher). Final destination vectors were amplified in *E. coli* and then purified using the extraAxon plasmid mini kit (Axonlab). The destination vectors were then transformed into *Agrobacterium tumefaciens* strains GV3101 and LBA4404 and grown on solid selective YEB medium at 28 °C for 2 days before being used for subsequent stable plant transformation.

Stable transformation

P. axillaris N and *P. axillaris* P leaves were transformed using *A. tumefaciens* (strains GV3101 or LBA4404) carrying the respective overexpression construct. *A. tumefaciens* cultures were grown for 2 days on solid media (YEB media with appropriate antibiotics rifampicin, streptomycin, spectinomycin or gentamycin) at 28 °C before inoculating 5 ml YEB liquid medium (with appropriate antibiotics streptomycin, spectinomycin or gentamycin) with *A. tumefaciens* cultures to grow overnight at 28 °C. After centrifuging the overnight cultures at 3500 rpm for 15 min, the liquid supernatant was discarded and the pellet resuspended in 40 ml of autoclaved MilliQ water with 0.01 mM acetosyringone to aid *A. tumefaciens* infection. Liquid cultures were kept in the dark at room temperature until used for plant inoculation. Leaves from 4-6 week old plants of both *P. axillaris* N and P were sterilized in a 10% hypochlorite solution for 10 min and rinsed five times with autoclaved MilliQ water. The sterilized leaves were then cut into 1 cm^2 pieces and inoculated in the *A. tumefaciens* suspension for 30 min (in the dark with shaking). The leaf fragments were then dried between two layers of sterilized filter paper and transferred to solid Murashige-Skoog (MS) growth medium without antibiotic selection. Leaf fragments were grown in the dark for 7 days at a constant temperature of 24 °C. After this first week, leaf pieces were then transferred to fresh selective growth medium containing Kanamycin to select for the presence of the overexpression construct. All plates were sealed with medical tape and incubated under the same temperature conditions and a light:dark regime of 16:8 h. Leaf fragments were transferred to fresh media once a week or more often if contaminations were detected for callus growth to be successful. Once shoots started to appear, each shoot was excised from the calli and transferred to MS rooting medium containing kanamycin for further selection of the construct. This allowed roots to form and shoots were transferred to soil (70% Klasman substrate, 15% Seramis clay granules, 15% quartz sand) when their roots reached a

length of 2-3 cm. Plants were then grown in the same growth chamber and under the same conditions as all other species (see section Plant Material for details). Screening for overexpression mutations was then performed on these plants after one week to allow them to acclimatize to the soil conditions.

DNA extractions and genotyping

Genomic DNA extractions were performed with fresh leaf samples using a modified CTAB (Murray and Thompson, 1980) or SDS protocol. For SDS extractions, samples were ground in 200 µl SDS extraction buffer and centrifuged at maximum speed for 10 min. The supernatant was then removed and mixed with 100 µl iso-propanol. After 10 min incubation, samples were centrifuged again at maximum speed for 10 min before the supernatant was discarded. Samples were washed with 100 µl 75% ethanol (centrifuged at maximum speed for 5 min), dried overnight and then resuspended with 100 µl water. All samples were analyzed with a Nanodrop ND-1000 (Thermo Fisher) prior to further analysis. *AN2* target sequences were amplified using Q5 polymerase (NEB) using the primers depicted in Suppl. Table S2.4. PCR reactions were run according to the manufacturer's protocol with the following parameters: annealing temperature 57 °C, elongation time 2:00 min with 35 cycles. Sequences were obtained through Sanger sequencing (GATC, Cologne, Germany and Microsynth, Balgach, Switzerland) with the primers listed in Suppl. Table S2.4. Purification of PCR samples prior to sequencing was done using the NucleoFast 96 PCR plate (Macherey-Nagel). All sequences were analyzed manually using the Geneious Primer 2020.2.4 software (Biomatters). Sequences of transformed plants were aligned to existing wildtype sequences of both *P. axillaris* accessions.

RNA extractions, cDNA preparation and quantitative RT-PCR

For each sample type collected, three individual flower buds (*Petunia* bud stage 4, 22-30 mm length) from four different plants, were collected (n=12 for each sample type, 4 sample types analyzed), representing biological replicates. The floral limb tissue of the buds was dissected and frozen immediately in liquid nitrogen. Tissue samples were stored at -80 °C until further processing. RNA extractions were performed using the innuPREP RNA Mini Kit 2.0. All plant tissue was first homogenized in liquid nitrogen before proceeding according to the manufacturer's protocol. Samples were analyzed with

a Nanodrop ND-1000 (Thermo Fisher) prior to further analysis. Before proceeding to cDNA synthesis RNA samples were DNase I (Sigma-Aldrich) treated to remove any residual DNA possibly present in the samples. cDNA was synthesized using the qScriber cDNA synthesis kit (Axonlab) and then used for RT-qPCR with the ORA SEE qPCR Green ROX L mix (Axonlab) according to the manufacturer's recommendations.

The expression levels of flavonoid pathway biosynthetic genes (Tornielli et al. 2009) were investigated. qPCR primers were used as published previously in Esfeld et al., 2018. Primer sequences for all genes are shown in Suppl. Table S2.4. All samples were compared to the three reference genes *SAND*, *RAN1* and *ACTIN11* (Mallona et al., 2010) by calculating the geometric mean of the relative expression of a gene compared to each reference gene. Primer efficiencies for each primer pair were determined using standard curves. RT-qPCR experiments were run on a QuantStudio 5, 384 well Real-Time PCR Machine (Thermo Fisher). All reactions were run in triplicate to calculate the means of the individual biological replicates using three data points. Using the QuantStudio Design and Analysis (v1.4.3) software the Cq values and calculations were performed. Values of the mutant and wildtype plants of each line were normalized to the three reference genes. For all analyzed biosynthetic genes statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons as data was normally distributed in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463. Results were considered significantly different if $p < 0.05$. Data was visualized using the ggplot2 package in R (Wickham, 2016).

Results

Restoration of a non-functional major effect gene via CRISPR/Cas9

To restore *AN2* function in *P. axillaris*, which has a non-functional copy of the gene, we used a CRISPR/Cas9 approach to induce a reading frame restoring 2 bp deletion, with the goal of recreating the strategy observed in *P. secreta*. Via CRISPR/Cas9 we targeted the early stop codon sequence in *P. axillaris*, to create an additional 2 bp deletion and restore color in the white species through a now functional *AN2* gene. Ideally the exact same 2 bp as in *P. secreta* would be deleted, but theoretically any 2+3n mutation would be capable of restoring the reading frame. However, if the mutation is too large this may cut out too many amino acids in the restored protein, compromising its function.

We designed four gRNAs to target the *AN2* gene in exon 3 (gRNAs 1, 2, 6 and 8) as close to the mutation site as possible while also minimizing predicted off-target effects (Fig. 2.1 A). gRNA 2 was the closest to the mutation site and preferred for transformations due to its position (Fig. 2.1 B). Using these four gRNAs, we constructed four gene constructs with single gRNAs as well as a combination of two gRNAs to make two double gRNA constructs (gRNAs 1/2 and 6/8). These constructs were used for stable transformation of natural *Petunia* species.

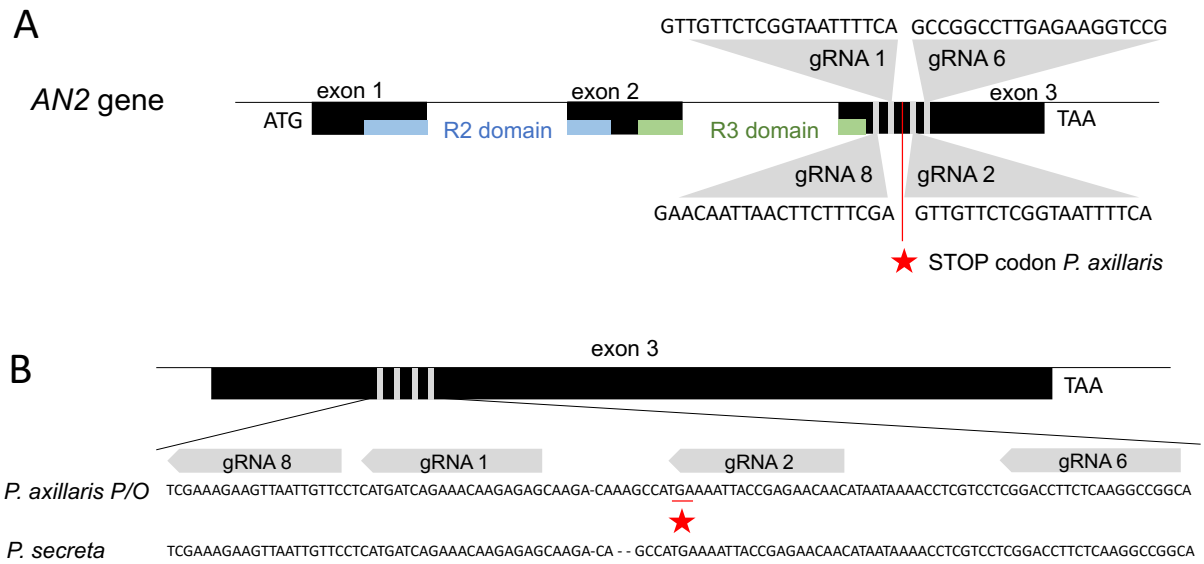

Figure 2.1: Designed gRNAs targeting exon 3 of the *AN2* gene. (A) Four gRNAs were designed around the premature stop codon in *P. axillaris*. (B) Detailed sequences of the designed gRNAs. The gRNAs are aligned to both the wildtype *P. axillaris* P/O and *P. secreta AN2* sequences with the additional 2 bp deletion in *P. secreta* shown between gRNA 1 and 2.

Mutation efficiencies vary with gRNA constructs

Since wild *Petunia* lines are recalcitrant to stable transformation, we set up stable transformations with various wild *P. axillaris* accessions (*P. axillaris* N, P and O) to establish a protocol optimized for wild *Petunia* (Table 2.1). *P. axillaris* P had the highest number of regenerated shoots (813 in total). All designed gRNAs led to mutations if shoots were regenerated from them. We were not able to obtain shoots from one construct, double gRNA 6/8, therefore also not obtaining any mutations with this construct. The mutation efficiency of the different gRNAs varied between 1.06 and 10.53 % of the regenerated plants with gRNA 6 generating the highest percentage of mutants (Table 2.1). No mutations were found in the *P. axillaris* lines N and O, most likely due to a

low transformation efficiency as not many plants were regenerated. These results demonstrate differences within the wild *Petunia* species, calling for optimization of protocols even for different lines of the same wild *Petunia* species.

Table 2.1. Regenerated plants with gRNA constructs targeting the *AN2* gene.

Plant species	gRNA construct	No. of transformed leaf discs	No. of regenerated plants	No. of plants with mutation	Percentage with mutation
P. axillaris P	gRNA 1	810	283	3	1.06 %
	gRNA 2	1005	341	22	6.45 %
	gRNA 6	510	57	6	10.53 %
	gRNA 8	465	82	3	3.66 %
	gRNA 1/2	945	50	2	4.00 %
	gRNA 6/8	915	0	0	0.00 %
P. axillaris N	gRNA 2	270	2	0	0.00 %
P. axillaris O	gRNA 2	60	11	0	0.00 %
Total		4980	826	36	4.36 %

Both insertions and deletions were obtained with the gRNA constructs, but mutations were relatively short in length (1-10 bp) and located at the 5' end of the gRNA target. gRNA 6 did not lead to mutations that would restore the reading frame as it was located downstream of the premature stop codon found in *P. axillaris* (Fig. 2.1 B). gRNAs 1 and 8 did not give rise to any 2+3n mutations capable of restoring the reading frame.

Premature stop codon cannot be eliminated via CRISPR/Cas9

gRNA 2 was the most promising in our setup as it was the closest to the 2bp target site. However, restrictions in the gRNA design based on the plant-specific Cas9 enzyme placed the 5' end of the gRNA at the stop codon (Fig. 2.1 B). Following this premature stop codon are three additional adenines, replacing the last adenine of the stop codon if only 2 bp are cut out at this site. Analyses of the sequences obtained with gRNA 2 led to three mutations that were either very close to restoring the reading frame but did not eliminate the premature stop codon, or were not a 2+3n mutation but able to remove the stop codon (Fig. 2.2). As none of the obtained mutations in the 826 plants we screened restored the

reading frame, we did not observe any phenotypic differences in the transformed plants. Thus, we did not recapitulate the precise mutation observed in *P. secreta* that restores the *AN2* reading frame in *P. axillaris.*

```
                                        gRNA 2
P. axillaris P   GAGCAAGACAAAGCCATGAAAA-TTACCGAGAACAACATAATAAAACCTCGTC
P. secreta       GAGCAAGACA - - GCCATGAAAA-TTACCGAGAACAACATAATAAAACCTCGTC
1 bp ins.        GAGCAAGACAAAGCCATGAAAAATTACCGAGAACAACATAATAAAACCTCGTC
5 bp del.        GAGCAAGACAAAGCCATGAA - - - - - -CCGAGAACAACATAATAAAACCTCGTC
9 bp del.        GAGCAAGACAAAGC - - - - - - - - - - TACCGAGAACAACATAATAAAACCTCGTC
```

Figure 2.2: *AN2* mutations obtained with gRNA 2. Three mutations are shown that either restore the reading frame (1 bp insertion and 5 bp deletion) or eliminate the stop codon colored in red (9 bp deletion). None of the mutations obtained were able to simultaneously restore the reading frame while also eliminating the stop codon.

Expression of the *AN2* gene under *CaMV35S* and native promoters partially restores visible color

Obtaining a phenotypic difference in *P. axillaris* flowers via the CRISPR/Cas9 approach was challenging for two reasons: first of all obtaining a precise mutation to restore function of the *AN2* gene proved to be relatively difficult. Additionally expression of the *AN2* gene varies substantially between *P. axillaris* lines, ranging from low expression (Esfeld et al., 2018) to levels exceeding those of the short tube clade (Hoballah et al., 2007), potentially influencing restoration of the visible color phenotype even if repairing the *AN2* gene is successful. These challenges become obsolete with transgenic approaches, which is why we undertook a classical transgenic approach to restore color in *P. axillaris* P. We first used the *CaMV35S* promoter to express the *P. secreta AN2* gene in a *P. axillaris* P background (Fig. 2.3 A). This led to light pink floral limbs as well as visible purple pigmentation in the vegetative organs as the *CaMV35S* promoter is ubiquitously expressed (Fig. 2.3 B). We then attempted to use the petal-specific native *AN2* promoters of *P. secreta* and related *AN2*-functional species *P. inflata* to express *AN2* (Fig. 2.3 A). This led to a similar light pink floral phenotype for the *P. inflata* promoter while the *P. secreta* promoter produced an almost white phenotype (Fig. 2.3 B). No differences in the color of the vegetative organs were visible.

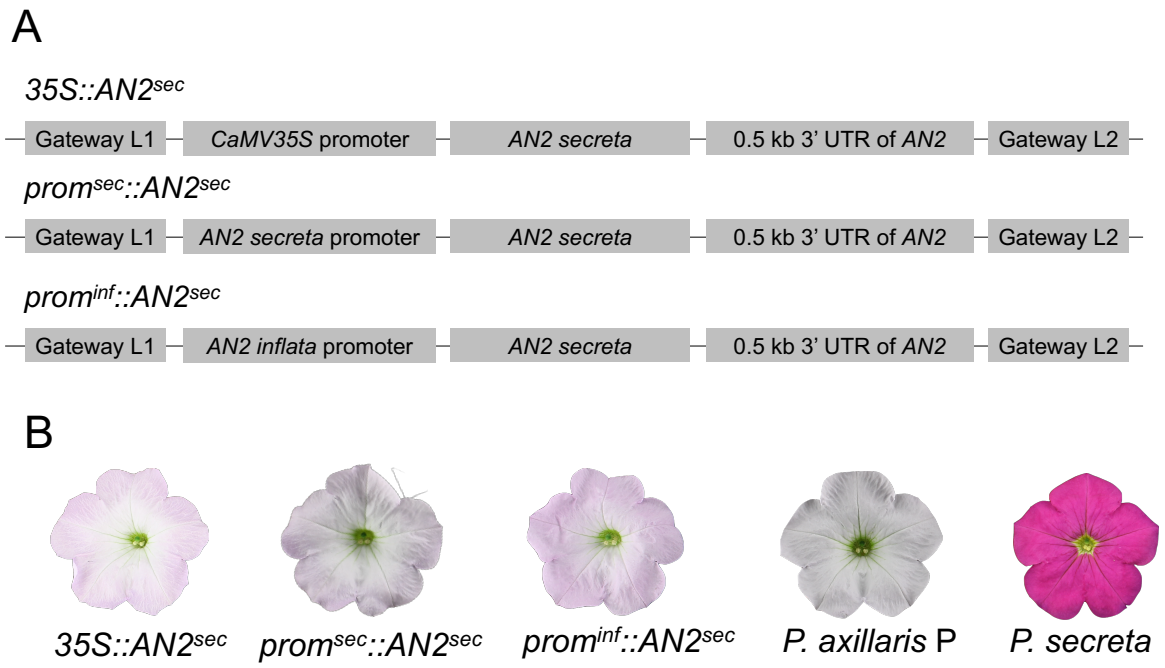

Figure 2.3: **Gene constructs for transgenic complementation with different promoters.** (A) The *CaMV35S* promoter as well as the native *AN2* promoter of *P. secreta* and *P. inflata* were used for expression of the *AN2* gene. (B) Visible color phenotypes of the different transformants compared to the wildtype *P. axillaris* P. All transformations led to light or faint pink phenotypes.

We expected that the observed phenotypic changes in floral pigment in our overexpression approach should be induced by alterations in the expression levels caused by the introduction of the *AN2* gene through different promoters. To test this hypothesis, we used an RT-qPCR approach to quantify flavonoid pathway gene expression. We analyzed the two major transcription factors *AN2* and *MYB-FL* responsible for the production of visible and UV color, as well as further genes in the pathway (Fig. 2.4). Expression levels of the *AN2* gene were significantly higher in both *P. inflata* promoter and *P. secreta* promoter lines, similar to the levels of *AN2* expression in wildtype *P. secreta* (full statistics for RT-qPCR data in Suppl. Table S2.1). *MYB-FL* was significantly downregulated in both transgenic lines, at an intermediate level between the *P. axillaris* P and *P. secreta* wildtypes. The only other two genes that showed significant differences between the transgenic lines and the *P. axillaris* P wildtypes were *HT1* and *3'5'AMT*. Expression of these genes in the transgenic lines was lower than in *P. axillaris* P. No differences between the two transgenic lines (*prom^{inf}::AN2^{sec}* and *prom^{sec}::AN2^{sec}*) were detected in any of the analyzed genes. Although we observed

significant upregulation of the *AN2* gene at levels found in *P. secreta*, this gene alone was only able to restore weak coloration in a *P. axillaris* P background.

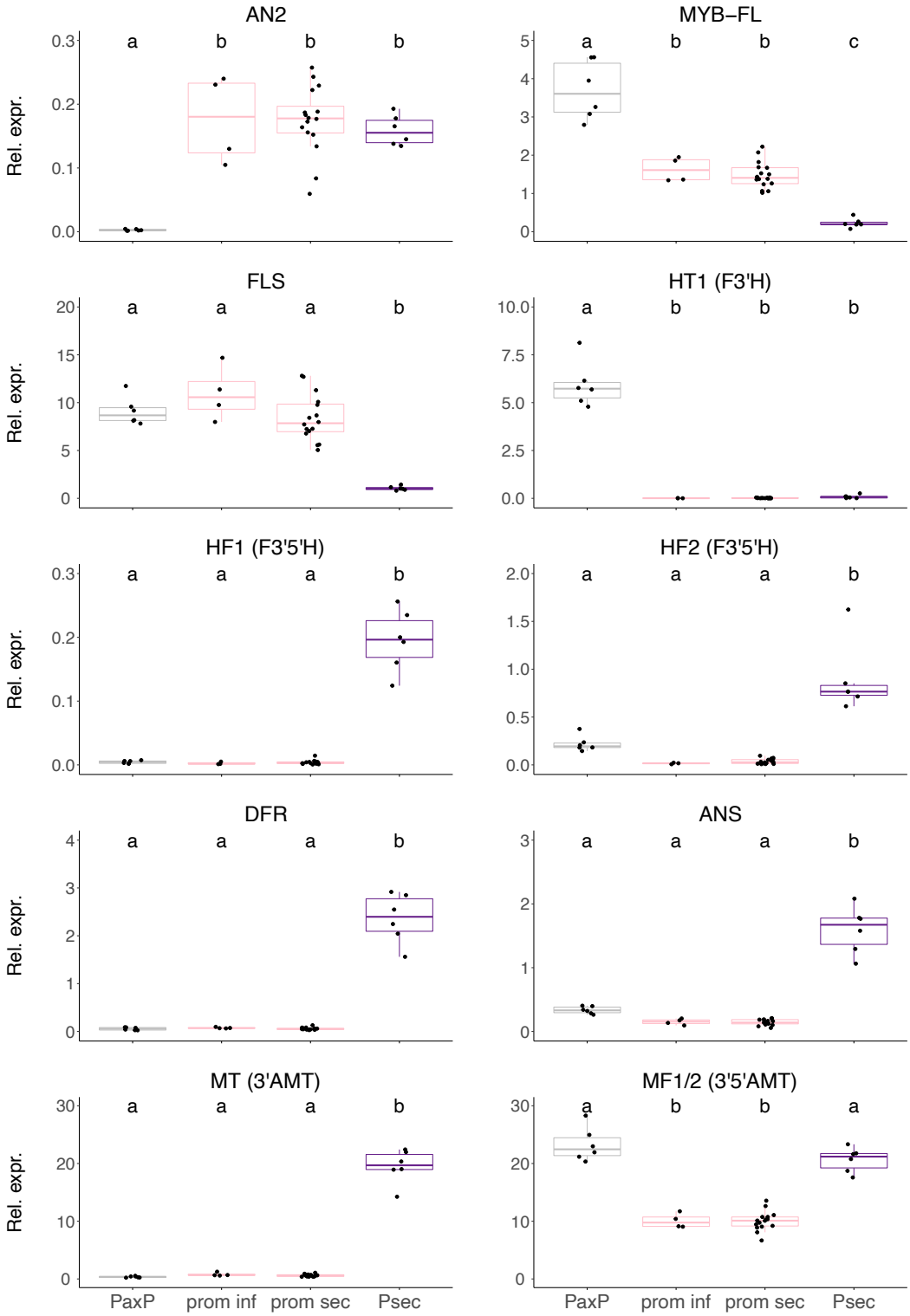

Figure 2.4: **Expression of genes influencing flavonoid pigmentation in native promoter lines.** RT-qPCR analysis of the different phenotypic colors obtained with the native promoter overexpression constructs (*prom*^{inf} and *prom*^{sec}) compared to the *P. axillaris* P and *P. secreta* wildtypes. Significant

differences in the expression of the *AN2* gene as well as other genes of the flavonoid biosynthetic pathway were detected. Expression is shown relative to the expression of the reference genes *SAND, RAN1 and ACTIN11*. Statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons and are depicted by letters (full statistics in Suppl. Table S2.1).

Strong petal-specific *CHS* promoters produce darker coloration

As neither the *CaMV35S* nor the native *P. secreta AN2* promoter led to the predicted purple color as in *P. secreta*, we created constructs using two chalcone synthase (*CHS*) promoters from two *Petunia CHS* homologs (*CHS-A*, 3kb and 800bp, and *CHS-J,* 3kb) that are strong petal-specific promoters (van der Meer et al., 1990; Liu et al., 2011) (Fig. 2.5 A). Two different flower color phenotypes were observed for transformants with the *CHS-A* promoter. Some of these transformed plants displayed a darker pink color after transformation with *pCHS-A* than with the native promoters used previously and some displayed a similar light pink phenotype (Fig. 2.5 B), possibly due to differences in the insertion sites of the transgene in the genome. A similar light pink phenotype as with the native promoters was also found for *pCHS-J*. No transformants were obtained with the shorter *CHS-A* promoter.

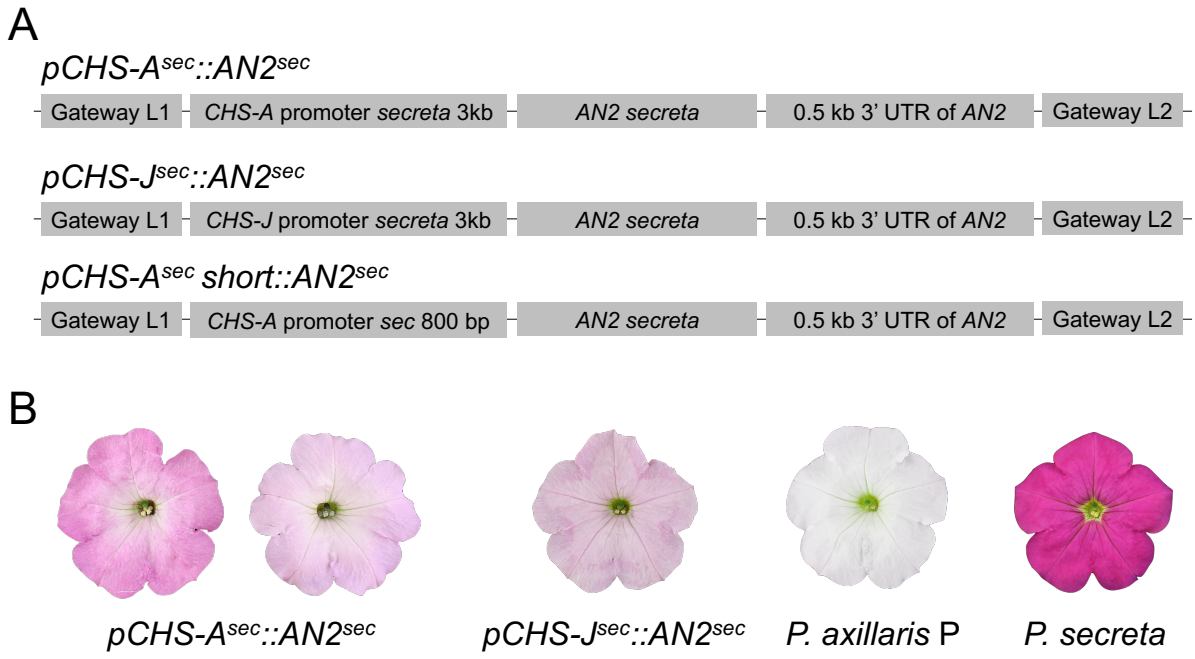

Figure 2.5: **Gene constructs for transgenic complementation with different *CHS* promoters.** (A) *CHS-A*, *CHS-J* and a shorter *CHS-A* of *P. secreta* were used for overexpression of the *AN2* gene. (B) Visible color phenotypes of the different transformants compared to the wildtype *P. axillaris* P and *P. secreta* lines. The darkest pink phenotypes were obtained with the *CHS-A* promoter, but a lighter colored phenotype was also

obtained with this construct. The *CHS-J* promoter led to light phenotypes as well. No transformants were obtained with the shorter *CHS-A* promoter.

Expression of a functional *AN2* gene upregulates the expression of anthocyanin structural genes

To determine whether there were any detectable differences in flavonoid gene expression in the *pCHS-A:AN2*^{sec} and *pCHS-J:AN2*^{sec} transformants, we used RT-qPCR as described above (Fig. 2.6). All *CHS* promoter constructs had a significantly higher *AN2* expression than the *P. axillaris* P wildtype samples (full statistics for RT-qPCR data in Suppl. Table S2.2). Notably the expression levels of the *AN2* gene in the *pCHS-J* promoter and light pink *pCHS-A* promoter were at the levels of *P. secreta* while the expression levels of the *pCHS-A* transformants were significantly higher than in *P. secreta*. Even though expression levels of *AN2* were highly elevated, they were not able to fully restore visible color (Fig. 2.5). The expression levels of genes associated with the production of flavonols (*MYB-FL* and *FLS*) were significantly reduced in the colored transformants, pointing towards possible interactions of these genes when highly expressed. Expression levels of *DFR* and *ANS*, genes important for the production of anthocyanins, were significantly higher in the *pCHS-A* transformants but not in the *pCHS-J*. Expression levels of *HF1/2 (F3'5'H)* remained unaltered in most *pCHS* lines, except in *pCHS-A* producing the darkest pink phenotype (Fig. 2.6). Here *HF1* was significantly upregulated compared to *P. axillaris*, but was still significantly lower than in *P. secreta.* Overall the stronger changes in floral color in the *CHS* promoter lines compared to the native promoter lines can be explained by a combination of changes in gene expression: higher expression levels of *AN2* coupled with higher expression levels of other genes in the anthocyanin branch of the pathway (*DFR* and *ANS*), as well as a lower expression of flavonol related genes (*MYB-FL* and *FLS*).

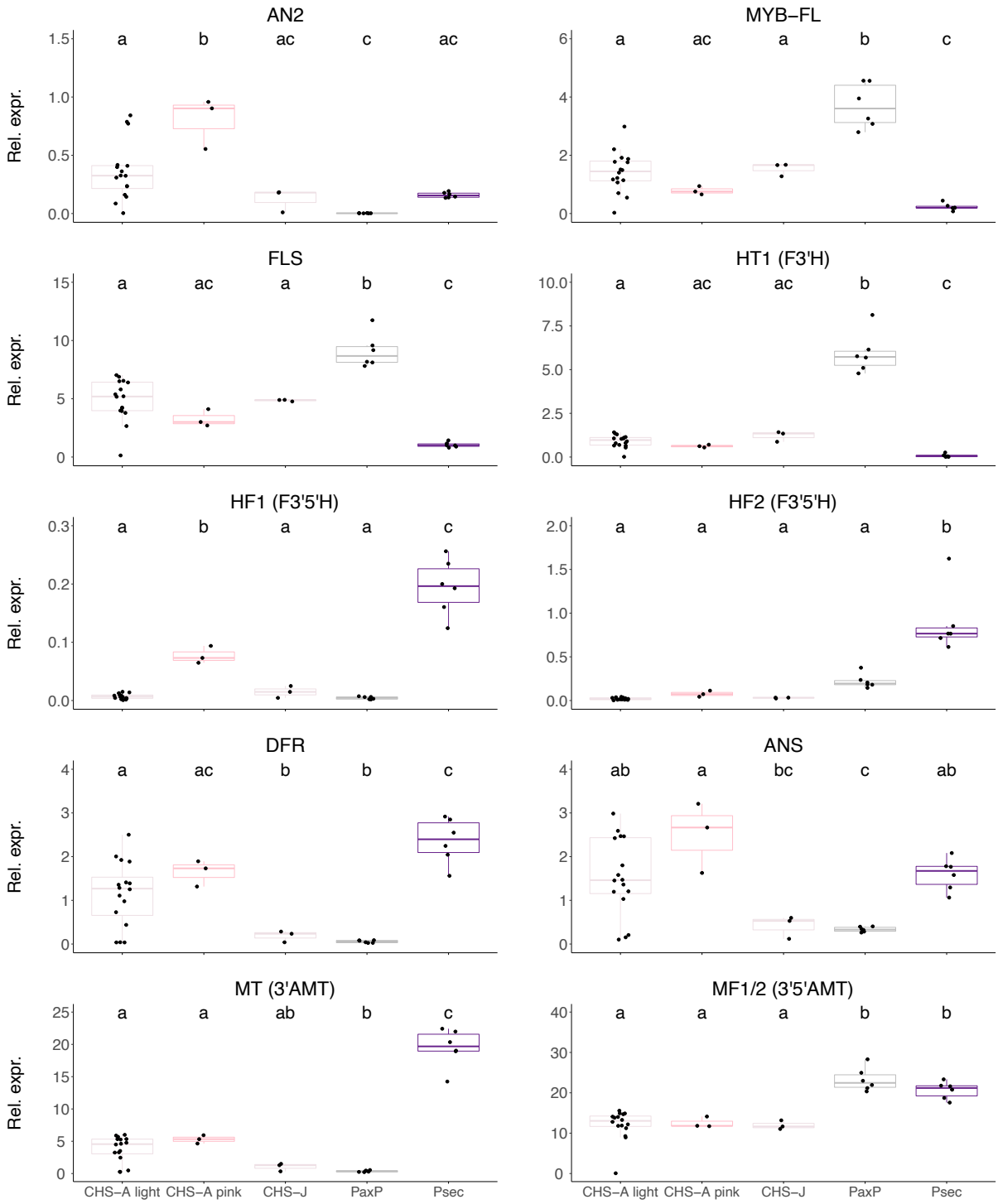

Figure 2.6: Expression of genes influencing flavonoid pigmentation in *CHS* promoter lines. Gene expression was analyzed via RT-qPCR to compare the *CHS-A* (light pink and pink phenotype) and *CHS-J* promoter overexpression constructs to the *P. axillaris* P and *P. secreta* wildtypes. Expression is relative to the expression of reference genes *SAND, RAN1* and *ACTIN11*. Letters display significant differences and were calculated using a one-way ANOVA with Tukey *post hoc* comparisons (full statistics in Suppl. Table S2.2).

Expression of a functional *AN2* gene leads to an increase in anthocyanidins

We also analyzed flavonol and anthocyanidin absorbance in the *pCHS-A* lines with the darkest pink phenotype and detected no difference in flavonol absorbance produced in the *pCHS-A* line compared to the *P. axillaris* P wildtype (Fig. 2.7, full statistics in Suppl. Table S2.3). The downregulation of the genes involved in flavonol production observed previously (Fig. 2.6) did not seem to be strong enough to affect flavonol production. However, we did find significantly higher anthocyanidin absorbance in the visibly pink colored overexpression lines compared to the white colored *P. axillaris* P wildtypes, showing that anthocyanidins are indeed responsible for the difference in visible color. Although the change in visible color phenotype was the strongest in *pCHS-A* lines, the visible color phenotype does not reach the deep magenta color of *P. secreta*. The cause for this phenotypic discrepancy is most likely the anthocyanidin absorbance in the *pCHS-A* lines that is, although elevated, lower than those in *P. secreta* (Fig. 2.7). This underlines the necessity of other enzymes of the flavonoid biosynthetic pathway being needed to restore complete visible color in *P. axillaris*.

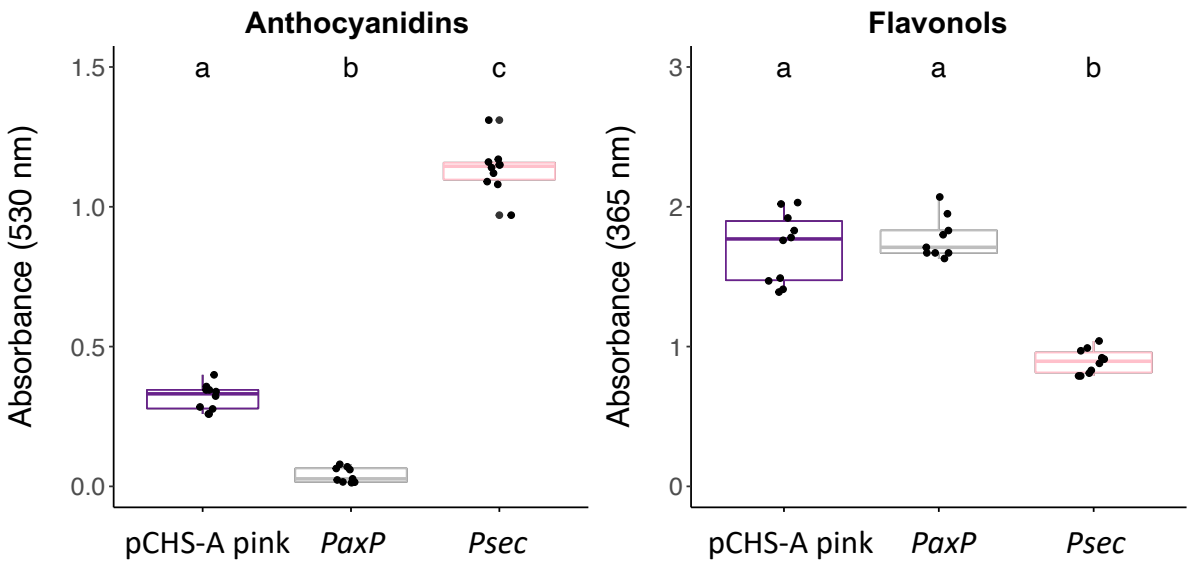

Figure 2.7: Flavonoid absorbance in the *pCHS-A::AN2^{sec}* lines. Spectrophotometric analyses were performed comparing the dark pink colored *pCHS-A::AN2^{sec}* overexpression lines to the white colored *P. axillaris* P and purple colored *P. secreta*. Data was analyzed using a one-way ANOVA with Tukey *post hoc* comparisons (full statistics in Suppl. Table S2.3). Significant differences are depicted by letters.

Discussion

The shift from hawkmoth to bee pollination syndrome in the long tube clade in *Petunia* involved the restoration of color in *P. secreta* (Esfeld et al., 2018). To investigate this regain of color, we aimed to restore function of the *AN2* gene in *P. axillaris* through different approaches. Expressing the *AN2* gene in a *P. axillaris* background using different promoters resulted in visible color restoration. However, the visible color phenotype in the transformants was not as strong as in wildtype *P. secreta* and although anthocyanidin absorbance values were elevated in the promoter lines, they were not restored to the same level detected in *P. secreta*. These findings demonstrate the effects of a single gene on visible color in *Petunia* and allow us to consider further factors that may contribute to the observed residual variation in phenotype.

Full, intense color cannot be achieved solely through restoration of *AN2*

We used multiple promoters to express the *AN2* gene in a *P. axillaris* P background attempting to restore full visible color: the *CaMV35S* promoter that exhibits transcriptional activity in various plant tissues at high levels (Amack and Antunes, 2020), native *AN2* promoters from both *P. inflata* and *P. secreta* as well as the strong petal specific *CHS-A* and *CHS-J* promoters (van der Meer et al., 1990; Liu et al., 2011). We defined full visible color as restoring anthocyanidin absorbance in *P. axillaris* to the same degree found in *P. secreta*. Visible color restoration was weak using the *CaMV35S* and native *AN2* promoters (Fig. 2.3) while *CHS* promoters induced a stronger change in visible color phenotype with the strongest regain in color being produced with the *CHS-A* promoter (Fig. 2.5). However, restoring *AN2* function through the flower specific *CHS-A* promoter did not restore full visible color as can be observed in wild *P. secreta* lines (Fig. 2.5) even with expression levels of the *AN2* gene being higher than in *P. secreta* (Fig. 2.6). Although anthocyanidin absorbance in the *pCHS-A* lines was elevated (Fig. 2.7), it was still lower compared to *P. secreta* (Esfeld et al., 2018). Expression of the *AN2* gene does not seem to be sufficient to completely restore color, even though the *AN2* gene is an R2R3-MYB transcription factor responsible for visible color pigmentation in *Petunia* (Quattrocchio et al., 1999; Hoballah et al., 2007; Esfeld et al., 2018). Our results are supported by previous work where transgenic complementation with the *AN2* gene also under the control of *CaMV35S* promoter showed similar phenotypic results to those

presented in this study with faint pink coloration of the *P. axillaris* lines (Hoballah et al., 2007; Esfeld et al., 2018). Near-isogenic lines (NILs), where the *AN2* locus was introgressed into a *P. axillaris* background, were also pink-purple colored, but less pigmented than *P. secreta* (Dell'Olivo and Kuhlemeier, 2013). There are multiple possibilities why these approaches may not have restored full color in *P. axillaris*.

A transcriptional trade-off between UV and visible color pigments may explain faint color

Both flavonols and anthocyanins are produced through the flavonoid biosynthetic pathway and share dihydroflavonol precursors (Fig. 1.4, Chapter 1). Due to their proximity in this pathway it is possible that a trade-off between the anthocyanin and flavonol branches of the flavonoid biosynthetic pathway influences pigmentation in *Petunia*. Trade-offs between flavonols and anthocyanins can either occur through substrate competition or through transcription factors. Previously, Sheehan et al. (2016) proposed a dual function for *MYB-FL,* the MYB transcription factor responsible for flavonol biosynthesis activation. *MYB-FL* acts as an activator of flavonol synthesis, but may simultaneously inhibit the production of anthocyanins (Sheehan et al., 2016). If such a trade-off exists, or at least some amount of anthocyanin inhibition, then *MYB-FL* activity will dampen any *AN2* activation, especially in a *P. axillaris* background where *MYB-FL* is highly expressed with high concentrations of flavonols. Given the lower but still elevated levels of *MYB-FL* expression we observed in transgenic lines with elevated *AN2* expression (Fig. 2.4 and 2.6) this could account for the partial coloration of the transgenic lines relative to the *P. secreta* wildtype lines. Additionally, competition for dihydroflavonol precursors needed for the flavonol and anthocyanin branches of the flavonoid biosynthetic pathway could influence the anthocyanin production in transgenic *AN2* lines, making them less pigmented. Rendering *MYB-FL* non-functional in our lines where *AN2* has been restored would help determine whether this restores visible color completely.

Downregulation of key enzymes in the flavonoid biosynthetic pathway hinder full color restoration

A further explanation for the partial restoration of visible color in *P. axillaris* through *AN2* expression, is differences in expression of other genes of the flavonoid biosynthetic

pathway that act downstream of *AN2* between colored and non-colored species. A combination of a functional and active *AN2*, low expression of *MYB-FL* and *HT1* (*F3'H*) as well as high expression of *HF1/2* (*F3'5'H*) makes the production of visible color in the colored species *P. inflata* and *P. secreta* possible (Esfeld et al., 2018). In *P. axillaris* on the other hand, *AN2* is inactive while *MYB-FL* and *HT1* (*F3'H*) are highly expressed (Esfeld et al., 2018). In our *pCHS-A* mutants we were able to integrate an active *AN2* and subsequently observed lower expression levels of *MYB-FL* and *HT1* (*F3'H*), similar to what is observed in *P. secreta* (Fig. 2.6). However, *HF1/2* (*F3'5'H*) were lowly expressed in the *pCHS-A* mutants (Fig. 2.6). Since *HF* directs the flow of the flavonoid biosynthetic pathway towards the trihydroxylated anthocyanins (delphinidin, petunidin, malvidin), this is a key enzyme involved in the restoration of color. While restoring *AN2* in a *P. axillaris* background induces anthocyanin biosynthesis, it is likely that only low quantities of trihydroxylated anthocyanins can be produced due to a low expression of *HF* and therefore may affect the visible color phenotype. Upregulation of these genes should restore their function as no loss-of-function mutations were detected in the coding sequence in *P. axillaris* (Berardi et al., 2021) and could be required to restore complete color in *P. axillaris*.

Analyses of the low amounts of anthocyanidins produced in *P. axillaris* reveals another important difference in the flavonoid biosynthetic pathway between colored and non-colored species of the long tube clade in *Petunia*. The composition of anthocyanidins in *P. axillaris* are contrary to what can be found in *P. secreta* (Esfeld et al., 2018). While purple colored *Petunia* species have the highest amounts of malvidin, followed by petunidin and very low levels of delphinidin, *P. axillaris* has the highest amount of delphinidin with almost no petunidin or malvidin detected, although overall anthocyanidin levels are very low compared to *P. secreta* (Esfeld et al., 2018). Since delphinidin is the precursor for the production of petunidin and malvidin, this indicates an issue with the anthocyanin methyltransferases in *P. axillaris* that catalyze this reaction. Indeed, Berardi et al. (2021) found that both copies of 3'5'AMT have premature stop codons in *P. axillaris* N. Changes in expression patterns of these genes in a *P. axillaris* background are therefore unlikely to affect visible color. With the absence of malvidin and petunidin, which confer more deeply purple hues, any color in a *P. axillaris* background may have a more light pink-purple hue. To achieve full color in *P. axillaris* it may thus be necessary to not only restore

AN2 function, but integrate functional copies of downstream enzymes of the pathway, *3'AMT* and *3'5'AMT* as well.

A role of fading in full color

Another locus to consider, that may affect the restoration of full color in *P. axillaris* is the *FADING* (*FA*) locus coupled with genes responsible for vacuole acidification. Acidification of the vacuole helps stabilize the stored anthocyanins (Passeri et al., 2016; Verweij et al., 2016). Certain genetic backgrounds with non-functional *ph4* or *ph3* genes (responsible for vacuole acidification) and a dominant *FA* allele trigger fading of the petal color through degradation of anthocyanins in the vacuole (de Vlaming et al., 1982; Quattrocchio et al., 2006; Verweij et al., 2016). Genotypes that accumulate peonidins, petunidins and malvidins display the most pronounced fading phenotypes (de Vlaming et al., 1982) and this phenotype always seems to be observed in combination with mutants of ph genes. In the wild *Petunia* species, the *FA* gene is the most highly expressed in *P. secreta,* a species that mainly produces petunidin and malvidin, and is more active in later stages of floral development (A. Berardi, unpublished data). *P. axillaris* produces overall low amounts of anthocyanidins almost exclusively composed of delphinidin, pointing towards two possibilities for the fading locus to influence visible color: (1) *P. axillaris* may actually produce higher amounts of methylated anthocyanins (peonidin, petunidin and malvidin) but these fade quickly making them difficult to detect or (2) The *P. axillaris* fading gene coupled with differences in vacuole acidification also affects the stability of delphinidin based anthocyanins. This is plausible because the accumulation of anthocyanin compounds decreases with increasing pH values (Zhang et al., 2014). Restoring anthocyanin levels in a *P. axillaris* background would therefore not display full purple color if the compounds are not stable in the vacuoles.

Restoring a gene is more difficult than breaking one

It is common sense that dictates that it is easier to break things than to repair them. This idea is also described in Dollo's law stating that the loss of complex features during evolution is irreversible (Marshall et al., 1994; Collin and Miglietta, 2008). Contrary to this belief, there are examples of trait and gene reversibility, for instance in *P. secreta* where the restoration of visible color was achieved through a surprisingly easy mechanism: a 2 bp deletion that restores the reading frame in *AN2* (Esfeld et al., 2018).

However, recreating this evolutionary event under laboratory settings was not as easy (Fig. 2.2). A combination of restrictions in the CRISPR/Cas9 gRNA design as well the gene sequence itself made it difficult to eliminate the premature stop codon. These difficulties could explain why multiple different *AN2* mutations that inactivate this gene have been found in natural populations, but only a single mutational event leading to the resurrection of *AN2* from a pseudogene background has been described (Esfeld et al., 2018). Additionally *P. axillaris* is the closest species to the common ancestor of the long tube clade that is currently available but has diverged itself over evolutionary time. Further key genes responsible for anthocyanin production in the flavonoid biosynthetic pathway were presumably no longer under selection and may have been degraded in *P. axillaris.* For instance HF1 and 2 (F3'5'H) were found to have extremely low expression levels in *P. axillaris* (Berardi et al., 2021) which may not have been the case in the common ancestor. This possible discrepancy between *P. axillaris* and the common ancestor may also account for the difficulties we encountered when trying to achieve trait reversibility.

The partially restored visible color phenotype we observed for *AN2* transgenic lines (Fig. 2.3 and 2.5) gives insight into the selection process that may have maintained this mutation in populations. If the chance of this restoring *AN2* mutation occurring is very low, it would need to induce a large enough fitness benefit to be maintained in populations. Since selection by pollinators in *Petunia* is strong and has led to floral diversification (Fregonezi et al., 2013), a switch in visible color under directional selection by pollinators would thus likely be maintained in a population. Behavioral studies with *AN2*$^{+}$ *P. axillaris* and wildtype lines showed no difference in preference for hawkmoths, but bees clearly preferred the *AN2*$^{+}$ lines (Dell'Olivo and Kuhlemeier, 2013). If this mutation restoring visible color conferred no negative benefits to the primary pollinator (hawkmoths) of *P. axillaris* but simultaneously was able to attract a new pollinator group (bees), even without full visible color, then it is highly probable that it was maintained in populations. Combining this change in color with other altered traits affecting pollinator preference will allow the shift from hawkmoth to bee pollination syndrome to be studied in even more detail.

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Supplemental data

Supplemental Table S2.1. RT-qPCR statistics of genes influencing flavonoid synthesis for transgenic complementation of *P. secreta AN2* through native promoters. P-values were calculated for relative expression analyses in *P. axillaris* and *P. axillaris* $prom^{inf}$*::AN2*^{sec} and $prom^{sec}$*::AN2*^{sec} overexpression lines (stage 4 bud limbs). As data was normally distributed statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons.

Gene	**Comparison**	**p-value** (adjusted with Tukey *post hoc* comparisons)
AN2	$prom^{inf}$ vs. *P. ax P*	0.0000141
	$prom^{sec}$ vs. *P. ax P*	0.0000001
	P. sec vs. *P. ax P*	0.0000129
	$prom^{inf}$ vs. $prom^{sec}$	0.9997681
	P. sec vs. $prom^{inf}$	0.9334772
	P. sec vs. $prom^{sec}$	0.8966238
MYB-FL	$prom^{inf}$ vs. *P. ax P*	0.0000002
	$prom^{sec}$ vs. *P. ax P*	< 0.0000001
	P. sec vs. *P. ax P*	< 0.0000001
	$prom^{inf}$ vs. $prom^{sec}$	0.9257978
	P. sec vs. $prom^{inf}$	0.0001264
	P. sec vs. $prom^{sec}$	0.0000075
FLS	$prom^{inf}$ vs. *P. ax P*	0.5226962
	$prom^{sec}$ vs. *P. ax P*	0.8849562
	P. sec vs. *P. ax P*	0.0000017
	$prom^{inf}$ vs. $prom^{sec}$	0.1437074
	P. sec vs. $prom^{inf}$	0.0000003
	P. sec vs. $prom^{sec}$	0.0000003
F3'H / HT1	$prom^{inf}$ vs. *P. ax P*	< 0.0000001
	$prom^{sec}$ vs. *P. ax P*	< 0.0000001
	P. sec vs. *P. ax P*	< 0.0000001
	$prom^{inf}$ vs. $prom^{sec}$	0.9999920
	P. sec vs. $prom^{inf}$	0.9961341
	P. sec vs. $prom^{sec}$	0.9914157
F3'5'H1 / HF1	$prom^{inf}$ vs. *P. ax P*	0.9993600
	$prom^{sec}$ vs. *P. ax P*	0.9999519
	P. sec vs. *P. ax P*	0.0000000
	$prom^{inf}$ vs. $prom^{sec}$	0.9996675
	P. sec vs. $prom^{inf}$	< 0.0000001
	P. sec vs. $prom^{sec}$	< 0.0000001
F3'5'H2 / HF2	$prom^{inf}$ vs. *P. ax P*	0.3060604
	$prom^{sec}$ vs. *P. ax P*	0.1084326
	P. sec vs. *P. ax P*	0.0000008
	$prom^{inf}$ vs. $prom^{sec}$	0.9972106
	P. sec vs. $prom^{inf}$	0.0000002
	P. sec vs. $prom^{sec}$	< 0.0000001
DFR	$prom^{inf}$ vs. *P. ax P*	0.9991752
	$prom^{sec}$ vs. *P. ax P*	0.9999985
	P. sec vs. *P. ax P*	< 0.0000001
	$prom^{inf}$ vs. $prom^{sec}$	0.9990474
	P. sec vs. $prom^{inf}$	< 0.0000001
	P. sec vs. $prom^{sec}$	< 0.0000001
ANS	$prom^{inf}$ vs. *P. ax P*	0.3107770
	$prom^{sec}$ vs. *P. ax P*	0.0872072

	P. sec vs. *P. ax P*	< 0.0000001
	prom^{inf} vs. prom^{sec}	0.9998382
	P. sec vs. prom^{inf}	< 0.0000001
	P. sec vs. prom^{sec}	< 0.0000001
3'AMT	prom^{inf} vs. *P. ax P*	0.9512631
	prom^{sec} vs. *P. ax P*	0.9754774
	P. sec vs. *P. ax P*	< 0.0000001
	prom^{inf} vs. prom^{sec}	0.9939923
	P. sec vs. prom^{inf}	< 0.0000001
	P. sec vs. prom^{sec}	< 0.0000001
3'5'AMT	prom^{inf} vs. *P. ax P*	< 0.0000001
	prom^{sec} vs. *P. ax P*	< 0.0000001
	P. sec vs. *P. ax P*	0.1187565
	prom^{inf} vs. prom^{sec}	0.9999892
	P. sec vs. prom^{inf}	< 0.0000001
	P. sec vs. prom^{sec}	< 0.0000001

Supplemental Table S2.2. RT-qPCR statistics of genes influencing flavonoid synthesis for transgenic complementation with *pCHS-A::AN2^{sec}* and *pCHS-J::^{AN2sec}*. P-values were calculated for relative expression analyses in *P. axillaris* and *P. axillaris pCHS-A::AN2^{sec}* and *pCHS-J::AN2^{sec}* overexpression lines (stage 4 bud limbs). Data was normally distributed and statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons.

Gene	**Comparison**	**p-value** (adjusted with Tukey *post hoc* comparisons)
AN2	*pCHS-A::AN2^{sec}* pink vs. *pCHS-A::AN2^{sec}* light	0.0071332
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2^{sec}* light	0.2865215
	P. ax P vs. *pCHS-A::AN2^{sec}* light	0.0035472
	P. sec vs. pCHS-A::AN2^{sec} light	0.1877853
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2^{sec}* pink	0.0011415
	P. ax P vs. *pCHS-A::AN2^{sec}* pink	0.0000149
	P. sec vs. *pCHS-A::AN2^{sec}* pink	0.0003635
	P. ax P vs. *pCHS-J::AN2^{sec}*	0.8912520
	P. sec vs. *pCHS-J::AN2^{sec}*	0.9989293
	P. sec vs. *P. ax P*	0.6131009
MYB-FL	*pCHS-A::AN2^{sec}* pink vs. *pCHS-A::AN2^{sec}* light	0.4521486
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2^{sec}* light	0.9981330
	P. ax P vs. *pCHS-A::AN2^{sec}* light	0.0000001
	P. sec vs. pCHS-A::AN2^{sec} light	0.0022031
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2^{sec}* pink	0.5474432
	P. ax P vs. *pCHS-A::AN2^{sec}* pink	0.0000014
	P. sec vs. *pCHS-A::AN2^{sec}* pink	0.6868050
	P. ax P vs. *pCHS-J::AN2^{sec}*	0.0001785
	P. sec vs. *pCHS-J::AN2^{sec}*	0.0331904
	P. sec vs. *P. ax P*	< 0.0000001
FLS	*pCHS-A::AN2^{sec}* pink vs. *pCHS-A::AN2^{sec}* light	0.4224017
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2^{sec}* light	1.0000000
	P. ax P vs. *pCHS-A::AN2^{sec}* light	0.0000113
	P. sec vs. pCHS-A::AN2^{sec} light	0.0000578
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2^{sec}* pink	0.6706133
	P. ax P vs. *pCHS-A::AN2^{sec}* pink	0.0000352
	P. sec vs. *pCHS-A::AN2^{sec}* pink	0.2184736
	P. ax P vs. *pCHS-J::AN2^{sec}*	0.0023419
	P. sec vs. *pCHS-J::AN2^{sec}*	0.0070793
	P. sec vs. *P. ax P*	< 0.0000001
HT1	*pCHS-A::AN2^{sec}* pink vs. *pCHS-A::AN2^{sec}* light	0.9277355
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2^{sec}* light	0.9077805
	P. ax P vs. *pCHS-A::AN2^{sec}* light	< 0.0000001
	P. sec vs. pCHS-A::AN2^{sec} light	0.0346896
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2^{sec}* pink	0.7029289
	P. ax P vs. *pCHS-A::AN2^{sec}* pink	< 0.0000001
	P. sec vs. *pCHS-A::AN2^{sec}* pink	0.6554786
	P. ax P vs. *pCHS-J::AN2^{sec}*	< 0.0000001
	P. sec vs. *pCHS-J::AN2^{sec}*	0.0573598
	P. sec vs. *P. ax P*	< 0.0000001
F3'5'H1 / HF1	*pCHS-A::AN2^{sec}* pink vs. *pCHS-A::AN2^{sec}* light	0.0001296
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2^{sec}* light	0.9773250
	P. ax P vs. *pCHS-A::AN2^{sec}* light	0.9989907
	P. sec vs. pCHS-A::AN2^{sec} light	< 0.0000001
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2^{sec}* pink	0.0094538
	P. ax P vs. *pCHS-A::AN2^{sec}* pink	0.0003589

	P. sec vs. *pCHS-A::AN2*^{sec} pink	0.0000001
	P. ax P vs. *pCHS-J::AN2*^{sec}	0.9566806
	P. sec vs. *pCHS-J::AN2*^{sec}	< 0.0000001
	P. sec vs. *P. ax P*	< 0.0000001
F3'5'H2 / HF2	*pCHS-A::AN2*^{sec} pink vs. *pCHS-A::AN2*^{sec} light	0.9802782
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2*^{sec} light	0.9999822
	P. ax P vs. *pCHS-A::AN2*^{sec} light	0.0980787
	P. sec vs. pCHS-A::AN2^{sec} light	< 0.0000001
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2*^{sec} pink	0.9962396
	P. ax P vs. *pCHS-A::AN2*^{sec} pink	0.7067495
	P. sec vs. *pCHS-A::AN2*^{sec} pink	0.0000008
	P. ax P vs. *pCHS-J::AN2*^{sec}	0.4549456
	P. sec vs. *pCHS-J::AN2*^{sec}	0.0000003
	P. sec vs. *P. ax P*	0.0000007
DFR	*pCHS-A::AN2*^{sec} pink vs. *pCHS-A::AN2*^{sec} light	0.6589083
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2*^{sec} light	0.0927289
	P. ax P vs. *pCHS-A::AN2*^{sec} light	0.0043097
	P. sec vs. pCHS-A::AN2^{sec} light	0.0013786
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2*^{sec} pink	0.0348974
	P. ax P vs. *pCHS-A::AN2*^{sec} pink	0.0049761
	P. sec vs. *pCHS-A::AN2*^{sec} pink	0.4315458
	P. ax P vs. *pCHS-J::AN2*^{sec}	0.9977300
	P. sec vs. *pCHS-J::AN2*^{sec}	0.0001100
	P. sec vs. *P. ax P*	0.0000016
ANS	*pCHS-A::AN2*^{sec} pink vs. *pCHS-A::AN2*^{sec} light	0.2066195
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2*^{sec} light	0.1103626
	P. ax P vs. *pCHS-A::AN2*^{sec} light	0.0102279
	P. sec vs. pCHS-A::AN2^{sec} light	0.9996898
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2*^{sec} pink	0.0083560
	P. ax P vs. *pCHS-A::AN2*^{sec} pink	0.0012424
	P. sec vs. *pCHS-A::AN2*^{sec} pink	0.3751757
	P. ax P vs. *pCHS-J::AN2*^{sec}	0.9998335
	P. sec vs. *pCHS-J::AN2*^{sec}	0.1483591
	P. sec vs. *P. ax P*	0.0307415
3'AMT	*pCHS-A::AN2*^{sec} pink vs. *pCHS-A::AN2*^{sec} light	0.7231361
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2*^{sec} light	0.1727582
	P. ax P vs. *pCHS-A::AN2*^{sec} light	0.0061612
	P. sec vs. pCHS-A::AN2^{sec} light	< 0.0000001
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2*^{sec} pink	0.0724610
	P. ax P vs. *pCHS-A::AN2*^{sec} pink	0.0080880
	P. sec vs. *pCHS-A::AN2*^{sec} pink	< 0.0000001
	P. ax P vs. *pCHS-J::AN2*^{sec}	0.9863324
	P. sec vs. *pCHS-J::AN2*^{sec}	< 0.0000001
	P. sec vs. *P. ax P*	< 0.0000001
3'5'AMT	*pCHS-A::AN2*^{sec} pink vs. *pCHS-A::AN2*^{sec} light	0.9992652
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2*^{sec} light	0.9999951
	P. ax P vs. *pCHS-A::AN2*^{sec} light	0.0000003
	P. sec vs. pCHS-A::AN2^{sec} light	0.0000323
	pCHS-J::AN2^{sec} vs. *pCHS-A::AN2*^{sec} pink	0.9992966
	P. ax P vs. *pCHS-A::AN2*^{sec} pink	0.0003355
	P. sec vs. *pCHS-A::AN2*^{sec} pink	0.0081735
	P. ax P vs. *pCHS-J::AN2*^{sec}	0.0001603
	P. sec vs. *pCHS-J::AN2*^{sec}	0.0040790
	P. sec vs. *P. ax P*	0.5880550

Supplemental Table S2.3. Statistics of flavonoid absorbance (spectrophotometer) for transgenic complementation with *pCHS-A::AN2^{sec}*. As data was normally distributed, statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons.

Flavonoids	Comparison	p-value
Flavonols	*P. axillaris* P vs. *P. secreta*	0.6854035
	pCHS-A::AN2^{sec} (pink) vs. *P. secreta*	< 0.0000001
	pCHS-A::AN2^{sec} (pink) vs. *P. axillaris* P	< 0.0000001
Anthocyanidins	*P. axillaris* P vs. *P. secreta*	< 0.0000001
	pCHS-A::AN2^{sec} (pink) vs. *P. secreta*	< 0.0000001
	pCHS-A::AN2^{sec} (pink) vs. *P. axillaris* P	< 0.0000001

Supplemental Table S2.4. Primers for RT-qPCR, PCR and cloning experiments.

Gene	Application	Primer sequences 5' – 3'	Citation
AN2	RT-qPCR	AN2_q4_F: GCATTGAGAAGTATGGAGAAGG	Esfeld et al., 2018
		AN2_q4_R: TGTGGCCTTAGATAATTCAACC	
MYB-FL	RT-qPCR	MYB-FL_qPCR_F: TACCACCACCACTACCACAG	Sheehan et al., 2016
		MYB-FL_qPCR_R: ACCTATCGCTGCTCCTGCAT	
FLS	RT-qPCR	NA625: CCAAGTTGAGATTVTTAGCAATGG	Albert et al., 2014
		NA266: ACCGGCCATGACATTCTTG	
HT1 (F3'H)	RT-qPCR	B582: GACTTCCGCCATGTCCGC	Esfeld et al., 2018
		B583: GCACACGTTCAATAACTGGCC	
SAND	RT-qPCR	SAND_F: CTTACGACGAGTTCAGATGCC	Mallona et al., 2010
		SAND_R: TAAGTCCTCAACACGCATGC	
RAN1	RT-qPCR	RAN1_F: AAGCTCCCACCTGTCTGGAAA	Mallona et al., 2010
		RAN1_R: AACAGATTGCCGGAAGCCA	
ACTIN11	RT-qPCR	ACT11_F: TGCACTCCCACATGCTATCCT	Mallona et al., 2010
		ACT11_R: TCAGCCGAAGTGGTGAAAGAG	
AN2	PCR	AN2_F: GCAGTGAGAACTATACATCATG	Esfeld et al., 2018
		AN2_R: TCTTCAATGGTCCCAATTAAC	

Supplemental Table S2.5. Gene fragment sequences for Gateway cloning of the *AN2* gene with different promoters.

Fragment	Sequence 5' – 3'
Psec promoter AN2	AAGGTAGCAGTGATTAGAATCAGATTTTCAGTAGAGTATGTGTGCAGATTTTCTTTAAGCTCAGAAAGACATACCTCTGCTGAATGAGCA GCTTCTTCTCCATCAGTTGCCATAAGAGCTGAGTCCTTCTTTTGGTCATCTTCTGTGTACATTTCTTTCAGCTTTTCCCACACTTCTTTGGC AGACTTGTATTCAAGTATCCTTGCTAGCACATCCGTAGTCAGCCCTTTCCTCAGGTGCAATTCAGCTTTGGAATTTTCCTGCAACCAGTTT AGGTCTTGAGGATCTAGCTCTTCTACCAGCTTTGGAACTAGAACCCCGTTTGCTGCTCTCTTTACAGGAACATGAGGTCCTCATTTCACAA TCAACCAGAGACTGTAATCCATTACTTGAAGGAAGTTTTTTCATCTATTTCTTCCAACACTTGTAGTTTGTCCCGTTAAACACAGGTGGCT TTGAGGGATCTCTTCCTCCTCTGAACTCCCGAGGATCTTCCTCGTCTGAACTCTCATCCGACAACCCCATCAGTGATAGTGGAGATTCATC TCCAGATTCTTTTTCATGATCAGATTCACTGTCAGTAGGTTCCCATGTAGCATAGAAAGCTTCCTTTCTCTTTCTTCTTTCTGCTCTGACG TTTGGAGGTCGTCTCACCTTCTTCTTATCTTTTTCATTCTCCTTTGACGCCTCCTTTTCCTTCTCATTTGTTGCTCTATTTTTCATCCATTC TACTTCCCACATTGGACAATCCATGATAGCATGGTCCTTCTTGAAACACTTGAAGCATCCTGAATAATTAGGACTATCAACTATGTTCTTT TTTCCAGAACTCTCCTTGTTATTTCTTTTGAACCCTTTGAACTTTCCCAAGTTAAACTTTTGTCTCAAAGCACTAACCATTGCTGCTTCAA CTGATTGCAAATCAACATAATCCTCAGTTTCATCAGTTGCTTCGAGTGCGAGGGTCTTGTCCTTCTTGAGAGCTGGTTTAGTTTTTGAATC TTTCATCATCTCATGTGTTTTCAAACTTCCAACCAGTTCTTCAAGTTTGATGGATCTGAGAGATTTGGACTCAATGATAGCAGTCATCTTG GGTTCCCAAGCCTCAGGTAACACATTTAGAACCTTATCCACCATCTCTACTTCAGGCAACTCCATATCTAGGATCTCATCTTATTAGTGAT AATGGTCAACCTGGTCATCATATCCTGGATTGATTTCTTCTCTCCCATAGAGAACAACTCAAACTGTCTTTTCAGCATTCTTCTCTTGGAG TCCTTCACTTCAGTGGTTCCTTCATGTGCAACTTGCAGGGCATCCCGGATTTCCTTACCAGTCTTATAAGATGATACTCTATTAAACTTCT CAGGGCCAAGACCACAAATTAACAGACGTTTGGCTTTGTGGTTCTTTTCAATCATCTTATAGTCCGCCTCATTGAACTCCTCTTTTGATTT AGGCTCTTGTCGGGAAGCAGCTCCTTCTCCAACTATTTTGGTAGGGATTAAATTCCCTTCAGATACTATAAGCCAGAGTCGGTAATCCTCA GCACAAATGTAGTTCTCCATTCTGTTCTTCCACCAGGCATAATACTGCCCATTGAACTGTGAGGGCCTTAGAACTGACTGTCCTTCAGTGA TGGTAGGTGGTGCACTCATGACTCTGATCCTTTTCAAGGTGTTAACCTAGCACGAGAAGACCTGCTCTGATACCACTTATTATAAACTAA GCACCTCCTAAGGACACTAGAAGGGGGGGTTGAATAGCATCTACCGATTTTTCTTTACGAGAACCTGATTCTCCTGGGTCAAGAACAAGT TCTTGACTTAGCAGTTTGCACTTTGACACCACAACACACAATCAAAAGGCACGAGGAACATGATGTTTTTACGTTAAAAACTCCTTGC TCAAGGGAAAAAAACCACAACCCACACTCTGAGATTTTCGAACCAATCCACTAACACAACGAGCAATTAGTTTCTGATTACAAGACTCTG TAACCAAGGGGCTACACTCTAGACCCTAATCACTCAACAAAGCTAACCTCTAGCTTTACAAAGTAACCTCTCAAAAGTTTACCTAGCACAA TTTGCTTCTATGATAAGCAGATAGGTAATAACAATGATAGCATAAAACTAGACAACTTACAAACCAAGAACCTTGACAGAATATCTAGAC TCAATCTACTTTAGCTCCACCAGTTGTCTTCTTCAGTTAAATCACGTTGAAAGTTGCTGAATCGCATGTGTATGCTCTCAACTCTCTTTTT GTGAATAACTGACTTGAGGCTGCATCAACTACTTGTATTTATATAGGGATCAGAACCATAGTTGAGTTCCACAAGGAAACCTTGTAAATA TGGCAAGACAAGATTTCCTATAACTCTTTACTTGTTTAAGTCCAACTCGTACCCATACTTCGAAGACACAGCTTGGGCACCACCTCTTTTA CTCTGACTAGGATGCATATTCGTTCACGCGTCACTACTCCTAATTCTGCAGGGAATTGCAACCCACAATCTTTTCGATCAGTCGTGCCCTT CTATACAAGGACCAGGTTCTCCTTCAAAACCACAGAGTTCTTCCCGTTTGTCATTCTTCAAAACGTTTCTTCTAACAACTTTAAATATTGA ACTTACCGAACTTAAATTTGTAAAGGTTTTTACACATTTATAGTTGTGATAATTTTCATGCAAGTTTGGTGATCCGTTGGTCACTGGTCA ACGTGTGGCCACGTTGTACTGTCGAGCATTTTCCAAAATCAATTCGTGTATATATATAGAGGGAGTGAAAGTTAATTGACTTCAAATCTG TGGGAAGATCTAGCAATAAGAAAGCCAGCAGGCTAGCTGCAGCTAGCTAGAGGTCTTTTCTAGTGGTGTCTTTGTGGTATATTTATTCTT TTTATTTCAATAATGCACCTCCTTTTAATTTACTTAAACTCAATTATGGAGCTTTCTTTGTCCTTTAGTTGTCAGTTGCAGTGAGAACTGT ACATC
Pinf promoter AN2	CAAGGGTATCGTCCGAAAGTCGTAGAGTCTGTTCTTTTGTTCACCCGCTCCAATCAACTTCCCCGTAGCCCGGTCCTGCATAACACACAAA TCATTAGTGAAATTTACATTGCACTCTAACTGATTAATCAATTGAGACACCGAAATCAAATTGCAAGTCATTTCGGGAACATATAACACT TTCTGAAGAACCATGTCTTCGTTAAAACTGAAGGATCCTTCTTTCGTAGCCATTGGACTGCTTCCGTTTGGTAGACCTACTGGACACAGTG GAATTGTCCGTAGATCCTCCAAAACACTCAATTGACCGGTCATATGATTTGATGCTCTCGTGCCAATAATCCACGGAAGTCGAATTTGCTT ACCAATCGATGGTTCACTTGTGCTTGTCTTCTCCTTGTTTAGCAGCTTGAACAACATCTGCAACTGATCACTTGTTATTCCTGACAAGTTA GTTTGTCCGGGCACAGTCATGTGTCCGGTCTCTCCACTTTCGGACTCAGCTTGAGCAGCATTTGCTCGATTTCCACTACGTCCTCGGACAG CATTTGAACCATACTGTTGATGTCCACGGCCTCTATCACCTCGGTTACCTTCAGCTCTTCCTCTACCACGTCCACCACTCAGAGAACCTTCT CTCGGATAATGAACCCAATCCTCCGGATAGCCAATAACTTCAAAGCAGCATTTGGGAGGATGTCCACCTCGATCGCACAAGTGACACTCCT TATAACCTTTCTTCGGTGGGAGCGACCGTTGTGCGACAAACGCTGTCACCTCCCCCCATTCTTCCAGAGTCCGTGCCATCCCCTTGACTCGC TCATCTTGAATTACCTTTTGGTAAACCTGATTCATCGATGGCAACGGATCCTGAGCCAAGACATTGGAACGGACGGTTCCATAAAGACTT TCATCCAACCCCATAAGAAATAAATGGACCCTATCTTCTTCTTTCTTCTTCTCCAGTGCAACCGTTAGATTGCATGTGCAATTTTCACACG AGAACGTTGGAAACTTATCATAATTCCCAAGTTCGTCCCACAACATAGCCAATTTTCCGTAGTAAGCAGCAACAGTCAATCCCTTTTGCTT ACAACCGGCCAAAGCATCTTTCAATTCCTGAATCTGAGGTCCGTTTCCCACTGAGAAACGCTTTCGAATACTCTTGCACAAATCTTTAGCA ACCTCCATATGCGATATGGTGGTCCTAAGAGTGTGCTCAATAGAATTCCTAATCCATGACACCAGAAGAGAATTGATAGTCCACCAATCC TCATGATCGACCGAATCTTCACCAGACTTCTTAATCGATCCATCAATGAAACCAAACTTCTTCCTTGCACACAGCACCGTTCTTATGGACC GTGCCCATTCTTCATACTCACCGGTCAACTGGACCTGTGTGATAATCAACCCAGGATTATCAGTCGAAGTGATATCGTACGGAGAGATGG TCTTCCGTACGACAGCAGCATCACCCCCACTCCTTGGACACCGCCTCCTCCACCAGCACCACCTATCGGAGCTGTCATTACTTTTCCACTCG AAGAGTAAAACGGGCACACCAACATAAAACTTAAGTGAAAACTCTTACACCTTATTGATGAATGAGTCCCCCTATTTATACAAGAAAAGT AGAAAACCCTATCTTGGGCATCAAGTATACTCCTAATGGGCTTTGCCCAATTAATTGTACAATTTACCCTAATTATATACACCTTACACAA ATAACCTATCCTGATATGATCTTAGGATCTAAGAATTGAAGAACAAACAAGGAATTAAAATAGATAGAAGATAGACACCAAATTTAAGA AATTGGAAGCAATGTTGGACTCAAACCAACACCTCTCCTTAGATTTCAAATAAAAGAGGCACCTAATCTAAAAAAACCCTCAAGAGGATT GGATACCTCTTGCAAGTTCATGGAAGTTTGGAAGCAACAATGACCTAGCCATAATCTCTCTCAAAGAGTTTTCACAATTCAACATAGTCT AATCTCAAAATGAAGACAACATAAACTATTTATACTAGGGCTTAATTAAAACTAACAATTACTTAAATAGCCCTAGAGCTAGTTATGACA AAAAGACTAAAAGTCTACTTCCGCTCCAATTTGCTCTTTTTGGCCATTCTTGATTTGATCATTGGCTTCCAAGCCCTCTTGAAGCATTCCA AGCTTTTGAGGTCAACTCTAAATCTGCTAATGTCATGAGCGCCAATCCGGATCGCATCATATCCTACCTATACAATTGCACTAATAACCCC TAATATATTCTAATAAGTTATTAGAATCTAATATTGTCTTTTATGACAACCAAAAAAAATTGAAAAATCAAATCGAGCAAAATTGAAGT TAAAAGAGAAAAATCAAACCCACATGGAACTTATTTTAGTTCAGACTAGATGGAAGCTTTTCTAAAACTGAATGCACAGCCCTAGATGCA ATGCAGATATCTACAGCGTTATGGCACCGAGTTCACCTAAACCATGTACTTTCGATGCGTAAAATAAGTATAAATGTAAATACCTACTAA AAGTGTAACAAATAATAGGAATGAACTCATAGTTTTAGATGTACCATCGATTCAATGTTAAAAACTTTAAATATTGAACTAACAGAACTT AAATTTGTAAAGGTTTTTACACATTTATAGTTGTGGTAATTTTCATGCAAGTTTGGCTATCCGTTGCAAGTTTGGCGATCCGTTGGTCAC TGGTCAACTTGTGGCCACGTTGTACTGTCGAGCATTTTCCAAAATCAATTCGTGTATATATATATAGAGGGAGTGAAAGTTAATGACTTC AAATCTGTGGGAAGATCTAGCAATAAGAAAGCCAGCAGGCTAGCTGCAGCTAGCTAGAGGTCTTTTCTAGTGGTGTCTTTGTGGTATATT TATTCTTTTAATTTCAATAATGCACCTCCTTTTAATTTGCTTAAACTCAATTATGGAGCTTTCTTTGTCCTTTAGTTGTCAGTTGCAGTGA GAACTATACATC
Psec CHS-A promoter	TCACTATCTACTACTTTCAAATATTATCCAACCTACCCACTAAATTATTTACCCACTCTACCCACCCAGACTTGTTTATGCAGTATACCAT AAAGGTATATACCTATAAGGCATAACAAAGGTATACAGTAATAAATGTCCCTCCAGACTTGTATACCATAATTTATGCAGTATACTTTAA AGGTATACACCTATAAGGCATAATAAAGGTATACAATAATAAATGGGTAGGTTGGGTAAATACTTTTAGTTTTATGGGTAGTATAATTT GTAATGGGTATGACTTGTAAATACTTTAAATTTTATGAATATAGAGTGTAAAAATCCCTATATTTAACTGCTCATACTTGAATAACAAGT TCACATTGTATATTTGTCAAAACTCACAAACTTCTAGATTAGATAGTGATAATTGTACAATTATCAATTATTCCATCCGTTCTATATTAT TTGATTTTTTGACTGTTTTCACACAGATTAAGGAATCCACATTTTAACATTAATTAAAGATGTCATTTACTAAATTAATCTTCATCTCTT TCAAAATTTACTCATAGAAAATTGCGAGAGATTTATGTGTTTAATTTATTAACAATGATAATTTAATTTAAAAAGTCAAAACAAATAAC ATCTTGGTTTTAAAAAAATTAAATATTTTGAACGCAAAAAAAAATTTAAAAATCATATAAAGTGAACCGGAGGGAGTATTAATTAAAA ATCGCCAACTAGTTACGATTTGGAGGTACTACTATTTTGATTTGGGAAGTACAAACCCTAACCTTTTTTGGAAACTTTAGCGGTTATGTT ATAAAAGAAAATTTAAAGAAGTTAGTACAGTTAGGTAAGCTATGAGGTATGGTTTTGGCCACGAGAGGGACCAATATGTCACGATATAA AAGTTAACTAATTCCTTTTGTTGCCCCTCAAATTAATTGATGTCACCTTCGTAAAACCTTGGTAGGAAATATTAGTTGTCGAGCATTCAA TTACAAAATATCTGTCTCTCCCTGGATGTACGTGTATCGGTCGGATTTTGTCGGGAAAAGTGCACGAAAGGCTACTGTTGTCACTGGTCTT TGGGCACAGGCCACTAATACCAAAATATTTAGTTTGTGGCCACTTTCGCTTAAAGAGATAGAACTTCAGTCCAAAGGCCGGATTGAAGTT CAGTCCTTAAAAATTGAACTTCAGTCCAGGGTCATATGGATTGAAGTTCACTCCTTAAAGACAGAACTTCAGTCCATGACCATGTTGACT GAAGTTCAGTCCTTAAAGATTGAACTTCAGTCCAGGGCCATATGGACTGAAGTTCAGTCCAGTCCTTAAAGATAGAACTTCAGTCCAGGA CCATGTTGACTGAAGTTCAGTCCAGTCCAGTCCTTAAGATGGAACTTCAGTCCAGGGCCATATGGACTGAAGTTCAATCCTTAAAGATGG AACTTCAGTCCAGTGCCATATGGACTGAAGTTCGGTCCTTAAAGATGGAACTTCAGTCCAGGGGTTGTATGGACTGAACTTCAGTCAATG ATCAGAATTTAAGTCAATATTTATTTAGTAAAGGCCCAAAAATGGCCTCTAATGTAAGACCAAAGAAAACAGTGGCCTCAAACCAAATAC TACTGTTAAAAGTGACTGCTCGGTGAAATTTTTATGATTTTGTCTGATCACATATATGAACACAGAGTCATAGTACGTATAACCATTTTC CTTACGCATGCAAAGCTCACATCCGTGTACTCAACAAAGTTACTGTAAATACAAAATTATACAATACTATACCAACGTGAAGA CCATGCAAATAAAAGTAGTGTCACATGTATGTATGATGCATTATTTATTTCAGTTTCTATTTGGACAAAACGAAAAGTATTGAAAACAAA ATTTTAAAGAGGCCACTGGGTATGTAAGACGTTCAAGTCTTGGTGTCCACACGTCAAACTTACTTAACCAATCTTCCAACACCACACAATT

	TACCTACTTAACAATCAAAAAGATCAAGAAATCCTTTTACCAAAAGAACCAGACCAAGAATATTCAACTATTATCATATTAGCTGTTATT GCTTGTATCACATAATATGCCTAACAACCTTTCATTTTACCAAACTCTACCTTCTTCAAATTTAATTTTCATTCATGCATCTAATGTACAA TAGTCCTTTTTATGACACCACACTCCAACCACCCAAATGAAATCCCCACCTCAAGTCTTAGCCCGACATAGTTTTTGGAGGGTTTTTTTTG AAAGTTTTTGCGTTTATATTTTTTGAGCAAAACTTGTCTCAAAAACTGGTCTCGAGCTGTTTTTGTCCAAAAACTAACATTTACACATAA AATTTCATTTTCATAAAAGCTCAGAGGCTCTTTTGAGCCAAAAAATTTGTTCCAAAAAAGTATGTCCAGACACTTTCATTTTCATATCAA ACTTCACCTAAAAAGTGTTTCCGAAAAAATATTTGGGAATCTATGTCCAAACACTATCTTAGACTAAATTCTCGAATAAATTAAATTAAA ATTTGTGTAAAACCAACATATGACCACAAATGTTAAAACATATAATGTGACACTGATGATGTATTAAATTTTTTACACTTTTAAATCACT TTTGTCTTTCGATCATAACGAAAAATGAAGAGTTTGAAACGCAAAAACAACAAAATTAACAGTTTTAGGCAGGGAGAGACACATTTGTTT GGAAAATATCTTCCAGAAAATAGTTACTCTAATGGAAATTAAACTTCATATATTGACAACTTAATTTTACTCAATTTGTTTCTATTATGT GACACAATTTTTTTACTAGACTCAGCTCCAACTTAGGCATTTTAAGTGCTTGTGCACTGTCAAATAGTGTCATAAAATTCAAAGGAGATA TATAGTAACTTAGAATTTACATATTCAAATATTCAAAAAGAGTGTTACAACTTGGTATTACGTGTCAAACTCCTGTTCAAAGCTGATGCT AGAAGTGACAGAAATCATATGTAAGAACGATCAAGACCATTCATTTGGTTCAACCAGTGGCGGATGTAGGGTGTTCACTGCGGGTTCGAT GGAATTCAGTAGGTTTGGTGCAGACAGTGTATTTATATCAAAAAAATTATTAAATATGTACAAAAATTAAGTTAGAGAATCCGTACTTG CACGTGAGATTGTTATACCAAAATTTAGAACTCATAAGGTTCAAATTCTAGATCCGGCTCCCGTTCAACAGATTGAAAAATCTCTGAGCC TTTGATATTGAGAAGTAGGTAGCGTTAATTCGAAAATAACCAACCATTAATTTGTGTTGAAGGTTTGCTAGGAAAATAAAAAAAGGATG TCACGTGTCATCAAGTTATTGCTACACGTGATTACTATCTACCATTCTCCTTTAGGGTTCTCATATAAATACTTTACAATCCCCATGCAAA CCATAACACAATATAAAAGATACTTACACTTGTCACGTACTACATAAAAAAAAAAAAATACCAAACTTTTTTCAAGCAAAA
Psec CHS-J promoter	GTCAAGCTTGAGGATTGCACTGCGCGCGCAGGAGCGACGCTTAGTGCTAGGCAAGGGACTGACATATATTTAGCCAACCCTGGGTGTGCG TATACTCAAGTTTTTGCATATGACATGCGCACAAGAGAGAAACCTAAGGGCGGCACAGTTGAAGCTAAGCACTGAGGCGAGCTTTTTAGA AAACTTGAGATGAGGAGCAGACTCTCTTGTAGTTAGGTAGGACTATGAGCACCGTACGGACTATTTGTGTGCCGCTATCATCGGAGAAGA GTAGTCCCGTCCTACGGGCGCCGCACATGCTATTTCAGCGCCGCACACGGGCTATTTGTGTTACGTGGTACAAGTACGCGCGCAGTACCTT TACAGAAGTAGCAGGTTAGTATCTTACAAGGTACTTTTGAAATAGAAAAAGAATCTTTTCCACACGAGTGATTAACAAGCTTAACACACC ACACGACAAAACTAATACGAAGGTAATTCTATTCGCTTTCTTTTGTAGGATATTGCTTTAATTAACTAACCTTGTTAATTTGAGAATTGTT TAAACAAATGTGAGAATGCATGCATGAAGTTTCAGTTCCAAATGAATCCATATTGTAGAATCAATATGTGGTTCTCTACATACTTTTAAA AGTCACGTTCTAAGACTAAAAAAAAAAAAAAAAAAAAAATTAGTTATGTGCTCTGACTGTAGAGAAATATTTTATGCTATCAAGTTAAGT ATCTACAACACAATGCGTGATCACATATGTATCAGCATGATATGACCGGCATTTGAACTAATAATGGTGTGGAACCTTTTAAATTAGTTA TAGGTTCATACTTAAATCCAAAAAAAAAAAAGCAGAAAATAATTGAGAGGTTCAGTTCTTTCTGTCTATGGTAAGAAAAGTCGTGGAGCA ACTTAGAACTAACACATATAAGAGGCTTTTATGGAGTCGTGGAGCAATTTAGAATCTAAGCCTGCAATCAATAGGTTTAGTGAGGAAATG AATATCAGGGCCTAACGAGTAAGTAGAATGAATATAAAGTCTGCTAAGAGAGTTCGTCATCCGATTAACATGAGAAATCAAAAACTCGGTG AAGAGTGGTTATTTGTTATGCTACATATGTAAAAGGGTAACGAGACGACGTTCTAACTTTCTCGTCCACATCTTATGCCAATCTTCCAAC ACCACAGAACACACCTACTTAACAATCAAACAAATAAGTGTGCCACTGTGCCTTGTTGTAAAAAAGTAGATATCAAGAAAAGAATCTTTA CAGATCCTCATCAAAATAGTTTGTTTGCGTGGAATACAAACCTAACTTCTTAATTTTCATTCGTACCAAACACAAGACTTTGTCCAAGAA TTTAATTTCACTCATGCGACTATGCAAGTACTGGTAATACTTTTCAGCCTAAAATGCTATTCGGTGCTCACACCCTAAAAATCATCTCCTA GAATCATTTTAAGTGTTTTGTTTTCTTTTATATCTTTCACAATTCAGAGTCATACGAGTAGAAGATTCAAGTGAACCGAGTGGGATCGCG GAGTAAATTTTTCTTACAATTCAAATAGTGGCGTCGCGTCTTGGATATTACAATTCTAACTTAAAATTATTGCATGCTAGTATATTGTAA CATGTTTGTATCACTATCCGAGTTTGGAATAGTTTAAAGGTACTTTTTTATTTACCAAATTAAAACTCTTAATTATACGTGTTGAACTCC TGTTATGTATTTGATTTTGAATTTCAGCCATGAGGGCCAAGAATCTCCCACAATCATATAAAAAAATGATAAGAATCGATATTTTAAATT TAATATCTAGACTCACCAATAGTACCCGAGATTAAAGATTATAATGGAATGGTAAAAATTCTTTATGGTTATTCAGAATTTTTGAGTTCG TGCTTCGAAAATGAAATTCCTTTTCTAGGAAGTAGGGGTGTTCACGGTTTGGTTTGGTTGGTTTTTGGTTAAAACCATAACCAAATCAAT ATATTCGGTTTTTAAATTCTTAAAAATCAAACGAAACCAAACCAAATAAAAGTTATATCCATCGGTTTGGTTATTGTTGGTTTGGTTCGA TTTTGTCAGGTTTAAAAAACAATATAACAATGAAAATAATTAGAATGATAAAAAGAGCTTTTCCTAAAACAATGATAAAGGGAATTTTT CTTAAAAATGAATGATAAAGAGAACTACAAGTATTTTCTTAGTCTTATAGTATTTTGAGCAACTTAAATTTTAATCTAATTATACCTAAA ATGTTGCATTCTTGCCACTACAACAAATCCCGGGGCAATTAAAAATAAATATTTAGAACAAAAGAAAGAATTAAAATATACTTATGTTTC TGCTTTCGATATGTCATGACTCATATATCAAGTTGAACATGCAATGGTTACTGAAAAGACGATGTCAAAACTAGAATTTGCTTTGCCTAG ACTTTATCGGTGCGTCTATGTGTCTGTGAGTCCGATTTTTTTTTACCTAAATTAATATAATTATAAGTTTATTGAATGAATTGGACTCCT TGGACTAAATGAGTTGGTTTCTTTTTAAAATGGACTTAAAAGTTAGCCACTCCTTGGACTAAATGTGTTGGTTTCTTGTTAAAATAGACT TAAAAGTTAGTTAAAAAGGTAAAATCTAATAACATTTAACCTTCTACAAAATTTATTGAAAACGTATAAAATACTTAATTACATTATAA AAAATTATTATATTAATATATATATAATTTTCCGGTTCGGTTTGGTTATTTTTCATGAAAACCAAATTAAATAATGTCGATTTTTAAAAT TTGAAAACCAAACCAAATCATATCCAAGAACGTATCGATTATTTTAATCGATTTGATTTGATTTTTGGTTTGGATCAGCTTTTAACCAAA CCGTAAACACCCCTTGGAAGTACCCAAATCCCGCATAAGAACTTCTATGATGTGGAGTTTTAATATATATATATATATATATGTGC GTGTGTGTGTGAGAGAGAAATATTTATAGTACTAATTTTTTACATGTATGTAACTCTTAAATAGTATAAAAAAACTGGTGACTTCACCAT TATCTACTACGCCGATTCGGATCCCGAACGGTGGGATGCATGGTTAATAAGGAGAAAAAGATTGACCAAAAGTATAACGTAAAGAAATTG ACCAAAAGCATAACGTAAAAAGATTGACCAAAACGTTTGACTTTGAGAAGTAGGTAGCGCAAGAAATCTCCCCTTGTAATTTTGGAAAAA GGAAATTGAGTTAATGATAAGAACCAAAGTCAAAAAAGGGATTATGAGAATTTATGAAAGAATACAATAACAGAAAAGATGAAAAATAT CGTGTGCCAACTGGGCTTAAAAAAGTTGCTCAAACACGTGAACACTAGCTACCAGTTATGAGATTTCTTCTATAAATACCAAACTCATGC ATACAAAGATCACTTCATTCTATTTTCTTTCACTAATAATACTTGCATACAATAGAGTTATATCCTTTTTTTTGTTGGCAACA
Psec CHS-A^{short} *promoter*	TTTGGAAAATATCTTCCAGAAAATAGTTACTCTAATGGAAATTAAACTTCATATATTGACAACTTAATTTTACTCAATTTGTTTCTATTA TGTGACACAATTTTTTTACTAGACTCAGCTCCAACTTAGGCATTTTAAGTGCTTGTGCACTGTCAAATAGTGTCATAAAATTCAAAGGAG ATATATAGTAACTTAGAATTTACATATTCAAATATTCAAAAAGAGTGTTACAACTTGGTATTACGTGTCAAACTCCTGTTCAAAGCTGAT GCTAGAAGTGACAGAAATCATATGTAAGAACGATCAAGACCATTCATTTGGTTCAACCAGTGGCGGATGTAGGGTGTTCACTGCGGGTTC GATGGAATTCAGTAGGTTTGGTGCAGACAGTGTATTTATATCAAAAAAATTATTAAATATGTACAAAAATTAAGTTAGAGAATCCGTAC TTGCACGTGAGATTGTTATACCAAAATTTAGAACTCATAAGGTTCAAATTCTAGATCCGGCTCCCGTTCAACAGATTGAAAAATCTCTGA GCCTTTGATATTGAGAAGTAGGTAGCGTTAATTCGAAAATAACCAACCATTAATTTGTGTTGAAGGTTTGCTAGGAAAATAAAAAAAGG ATGTCACGTGTCATCAAGTTATTGCTACACGTGATTACTATCTACCATTCTCCTTTAGGGTTCTCATATAAATACTTTACAATCCCCATGC AAACCATAACACAATATAAAAGATACTTACACTTGTCACGTACTACATAAAAAAAAAAAAATACCAAACTTTTTTCAAGCAAAA
AN2^{secreta}	ATGAGTACTTCTAATGCATCAACATCAGGAGTAAGGAAAGGTGCATGGACCGAGGAAGAAGATCTTTTATTGAGAGAATGCATTGAGAA GTATGGAGAAGGGAAGTGGCATCTAGTTCCGGTTAGAGCTGGTAATTTATTACTACTCCATCAGTTCCCTTATATAGGTGATCTTGTTTG ATTGGACACGAAATTTGAGAACAATATTTTTGTTTTAACTTGTTAATTTAAACATGTCATGATTTTTATGTCATTTAAATTTATTATTAA GTGTAAAATGAAAAGTTTAAGTTAAACTTGCCATGACCTTTATGTCATTTAAACCTGTTGTCACCGCATGAAGGCTAACTCCTAATATGA CTGTTTTGCTCTTTATATTCATGTTGAAGAAGTTTATATATGCAAGTGTTATACCACTGTATTTTATGTGAAATTTTGCAGGTCTGAATA GATGCAGGAAAAGTTGCAGACTTAGGTGGTTGAATTATCTAAGGCCACATATAAAAAGAGGGGACTTCTCTTTGGATGAAGTAGAGCTTA TTTTGAGGCTTCATAAGCTTCTAGGCAACAGGTAAGTTCAAGTTTGAACACTTGATGACCCTATTATCTCATAATTATATACACTCACCTT ATATTTATCTCAACCCTGAACTTCGCCTTTAGCATCTCTTCCCCAACCAAAGAAAAACTAACACTACTGAGAATCGAGATTTGCTCCATAG AAAGTCGTGGTTATTTTATTTTATTCATCGATTGTGTAAAATATATTTACATAATTAGTGTGGTTTCAAAATTTTTTATTTTTTACGAAG TTACCAATTATAGTTTATCATAAAGGTTTACTTGAAATTATCGTGTAACTGATTAGATTGCATAAATAATTTTGCACCATCAATGCCATA GAACATAAATTGCATCAGTAGGACTAGGATAGAACATAGATTCTTTCACTTAATATGCTTAAGTACTCGTCATTACCTCAAGTCTTTTAG ATGATCTAATAGAATAAAACCTTTACACTGTCAATACATAGAAATTTAATGCTTATTTTATGTTGGTTTAGATGGTCACTTATAGCTGGT AGACTTCCGGGAAGAACTGCAAACGATGTCAAAAACTATTGGAACACGCACCTTCGAAAGAAGTTAATTGTTCCTCATGATCAGAAACAA GAGAGCAAGACAGCCATGAAAATTACCGAGAACAACATAATAAAACCTCGTCCTCGGACCTTCTCAAGGCCGGCAATGAATCATGTTTCT TGTTGGAACGGCAAAAGTTGTAATAAAAACACTATAGACAAGAATGAAGGTGACACAGAAATAATAAAGTTTACTGATGAGAAGCAAAA ACCGGAAGAATCGATAGATGATGGACTTCAATGGTGGGCCAATTTATTAGCCAACAACATTGAGATTAAGGACTTAGCTAATGGTAATTC ACCAACATTGTTGCATGAAGAAATAGCACCATTGGTAAATATTGAGAGCAACCTCATGCAGGAAGGAGAAAGTGGCCTAAGTGACTTTTC AGTTGATATTGATGGCATATGGGATTTACTTAGTTAA
AN2^{sec} downstream (600 bp)	TTGGGACCATTGAAGAATTAATGAACTTGTAATAGAACTATAATTATACCTCTTTCTTTTTTACTTTATGTAAACGCTATAGTTGAAATC GTTATACATTTGAAATAATGGAATTACAATTATTATTTATTAAAAACTTAAAATATTTCGTTATGCATATTTCCTGTTTAAACAGTGAGC TAAATTGAATTCATTTTGAAAAAGATTGCAACAGCTCCTGTTCATTGTCGTGAAAGTGAAATCTGCTTGATTATGTGACACTGAATTTTT CATTTCACTTTGTTCAAAATAATGAGGCACTTCTGTTATAATCTATGCACCTTCTAAAGACACTAGAAGGGGGTTTGAATAGTGTTTACC GATTTTCGTTTTACGAACCTGGTTCTTCTGAATGTAGAATCAAGTTCTTACCTTCACAATTATACTGTTTTAACTACTGTAACATACCACA ACTACACACACAGAAAACACAAGGATTTTACATGGAAAACTCCTTGCTCAAGGGAGAAAAATCACGACCCACACTCTGGGATTTGAGAAC TGAATCCACTAACACAACGAGCAAGTTTCTGATTATAAACTCTGTAACCAAGGGGCTAC

Chapter 3

Single gene mutation in *MYB-FL* alters UV color in *Petunia* causing a major shift in pollinator preference

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Author contributions

Martina Lüthi: design of RNAseq experiment and behavioral assays; design of gRNA constructs for CRISPR/Cas9 gene editing; genetic manipulation of *P. axillaris* P *myb-fl* mutants; DNA extractions for sequencing of *P. axillaris* P; analysis of *P. axillaris* P *myb-fl* mutant sequences; phenotyping (UV/VIS photography, nectar volume/concentration, pollen germination, morphology, spectrophotometer measurements) of all *myb-fl* mutants; PTR-MS analyses of all *myb-fl* mutants; analysis of phenotypic data; confocal microscopy of epidermal cells; measurements of high light experiment; RNA extractions for RNAseq and RT-qPCR; RT-qPCR experiments and analyses; bioinformatic analyses of RNAseq data; behavioral assays with *M. sexta*, *B. terrestris* and *O. cornuta*; graphical visualization of all analyzed data; statistical analyses; writing of manuscript

Andrea E. Berardi: design of RNAseq experiment and behavioral assays; HPLC and spectrophotometric analyses; bioinformatic analyses of RNAseq data; reviewing of manuscript

Therese Mandel: genetic manipulation of *P. axillaris* N *myb-fl* mutants; DNA extractions for sequencing of *P. axillaris* N *myb-fl* mutants; analyses of *P. axillaris* N *myb-fl* mutant sequences

Loreta B. Freitas: provided plant materials

Cris Kuhlemeier: conceptualization; supervision

Abstract

To understand the molecular basis of speciation, identifying the major genes involved in speciation processes is key. The *MYB-FL* gene, encoding an R2R3-MYB transcription factor that regulates the biosynthesis of flavonol pigments, has been identified as a major determinant of floral UV color and pollinator preference in *Petunia*. In the white-flowered *P. axillaris,* the functional *MYB-FL* protein leads to an accumulation of flavonols and a UV-absorbing floral phenotype that attracts hawkmoths. *MYB-FL* was independently disabled in the sister taxa *P. secreta* and *P. exserta,* leading to UV-reflective flowers and associated pollinator shifts in each lineage (bees and hummingbirds, respectively). We created a *P. axillaris* mutant that represents a partial *P. secreta* mimic to study the individual steps of the speciation process and pinpoint the precise mutational path that led to their divergence. Through a CRISPR/Cas9 approach we genetically modified the *MYB-FL* gene, rendering it nonfunctional, to alter the floral UV phenotype from absorbent to reflective. Pollinator behavioral assays with hawkmoths and bees detected a strong shift in pollinator preference in this partial *P. secreta* mimic compared to the wildtype *P. secreta*. Furthermore, we evaluated the effects this single mutation had on flavonoid biosynthetic pathway gene expression using RNAseq. Knocking out *MYB-FL* function had specific effects on gene expression in this pathway: a reduction of target flavonol-related biosynthetic genes (*FLS* and *HT1*) as well as an induction of anthocyanin-related biosynthetic genes, which are two key adaptations in the *P. secreta* lineage. Ultimately combining *MYB-FL* with other major speciation genes to create a true *P. secreta* mimic, should demonstrate how many speciation genes are needed to obtain a complete shift in pollinator preference.

Introduction

Explaining the origin of species has been at the center of evolutionary research and remains one of the fundamental questions in biology. There has been a longstanding debate on how the process of speciation occurs. Darwin described the process of speciation as being gradual with small changes in phenotype while newer theoretical work shows that speciation through single mutational leaps is plausible if selection is strong (Orr and Coyne, 1992; Orr, 2005). From a genetic point of view, gradual change is caused through numerous mutations of small phenotypic effect while mutations in few genes of large effect can cause large phenotypic differences. Experimental evidence for both theories has been found (Doebley, 2004; Chan et al., 2010; Kooke et al., 2016; Nadeau et al., 2016), underlining why this has been a longstanding debate.

Speciation happening through gradual change was long thought to be the most plausible mechanism of speciation as small mutations allow descendants to slowly diverge from the parental population without removing it far from its fitness optimum as predicted through Fisher's theoretical work (Fisher, 1930). In new environments the descendants can therefore gradually adjust to the surroundings without major negative phenotypic effects. Large mutations on the other hand were predicted to remove a species far from their fitness optimum, most likely producing phenotypes with decreased fitness. In allopatry, long thought to be the predominant mode of speciation, this gradual mode of speciation may be plausible as the geographic barriers cause an instant cessation to gene flow (Coyne and Orr, 2004). Sympatric speciation, however, is difficult to connect with gradual change as gene flow is ongoing and recombination has homogenizing effects on populations. Therefore a central aim of contemporary speciation research is identifying the genes that differ functionally between species and how these influence ongoing speciation processes.

Establishing reproductive isolation barriers is an essential step during the speciation process. Isolation barriers act in a linear order, with prezygotic barriers occurring before postzygotic barriers. If multiple barriers are present, earlier barriers can thus contribute more to total isolation than the later barriers due to this linearity. This is why in flowering plants, reproductive isolation is often caused by prezygotic isolation mechanisms rather

than postzygotic barriers (Ramsey et al., 2003; Rieseberg and Willis, 2007; Lowry et al., 2008; Widmer et al., 2009; Dell'Olivo et al., 2011). Animal-mediated pollination is an important type of prezygotic barrier since plants are sessile and depend on vectors to disperse their pollen or seeds. Reproductive isolation can occur through differential visitation of pollinators due to diverging pollination syndromes, floral traits that are associated with the attraction of specific pollinators (Van der Niet et al., 2014).

As selection by pollinators is strong, the importance of genes with large phenotypic effects in pollinator-mediated selection has been demonstrated for several phenotypic traits (Hoballah et al., 2007; Rieseberg and Blackman, 2010; Sheehan et al., 2016). Flower color in particular is an important pollinator attraction trait, where studies have been successful in linking single genes or genetic loci to pollinator preference (Hoballah et al., 2007; Hopkins and Rausher, 2012; Yuan et al., 2013; Sheehan et al., 2016; Kellenberger et al., 2019). Considering how pollinators perceive floral color and distinguish between them is therefore important for understanding pollinator preference.

Depending on the geographical region, between 78% to 94% of flowering plants are pollinated by animals (Ollerton et al., 2011) demonstrating their importance for plant reproduction. Pollinators can perceive colors not only in the visible but also in the UV range (Chen et al., 1984; Kelber et al., 2003; Dyer et al., 2011; Stoddard et al., 2020). Considering how the pollinator visibility spectrum overlaps with the spectrum of pigments in a flower is important for understanding how pollinators perceive a specific floral display. Pollinators are able to distinguish and exhibit preferences between differences in both visible and UV color patterns (Hoballah et al., 2007; Dell'Olivo and Kuhlemeier, 2013; Papiorek et al., 2016; Moyroud et al., 2017; Stoddard et al., 2020). Certain pollinators, such as hawkmoths, prefer UV absorbing flowers while determining UV preference is more complex for other species, such as bees (White et al., 1994; Kevan et al., 2001; Sheehan et al., 2016). The detectability of a flower can be increased or decreased by UV reflectance depending on the reflectance that occurs in other parts of a bee's visibility spectrum (Kevan et al., 2001). Color contrast between the target and background seems to be more important for bees to detect a flower than the sole presence or absence of UV reflectance (Giurfa et al., 1996; Kevan et al., 2001). In fact for white colored flowers, UV reflectance can significantly prolong a bee's search time

(Spaethe et al., 2001) while for red colored flowers bees prefer UV reflecting flowers over UV absorbing ones (Chen et al., 2020). Both the visible and UV color display of a flower is therefore vital in determining pollinator visitation.

Floral color pigments are not only important for pollinator-mediated selection of plants, but can serve as a defense against different biotic and abiotic stressors in other plant organs, such as leaves (Gould, 2004). Abiotic stressors, such as geographic or bioclimatic factors, can also affect the occurrence of floral UV pigmentation and floral UV color diversification may be driven by non-pollinator-driven selection (Koski and Ashman, 2016). Species with UV patterns and overall smaller UV absorbing areas of the floral limb were found to grow in regions with lower UV-B irradiance than species with a higher total UV absorbing flower area (Koski and Ashman, 2016). Positive selection on UV pigmentation through UV irradiance (Koski and Ashman, 2015) shows that this observed correlation can also be demonstrated mechanistically. These differences in UV pigmentation across various altitudes can affect pollinator behavior and preference. Pollinator assemblages across altitudes can vary (Hodkinson, 2005) which results in shifting pollinator preferences affecting plant visitation. Additionally, the light environment in higher vs. lower altitudes may change how pollinators perceive visual signals. Bees displayed different degrees of color discrimination under different light regimes (Arnold and Chittka, 2012). Thus, determining how these differences in UV pigmentation due to abiotic stressors may affect pollinator preference is important to fully understand pollinator choices between species.

The major floral UV and visible color pigments responsible for pigmentation patterns are produced through the flavonoid biosynthetic pathway. Two main branches of this pathway produce the UV absorbing pigments called flavonols and the visible color pigments, the anthocyanins (Winkel-Shirley, 2001). Anthocyanins can range in color from red to purple to blue while the presence of flavonols produces UV absorbent flowers or UV patterns, not visible to the human eye. Synthesis of these compounds occurs as part of a complex metabolic network of phenylpropanoids that includes various compounds, such as defense compounds, lignins, volatile signals and developmental regulators (Winkel, 2006; Yang et al., 2017). In such large and complex transcriptional networks described in plants, promoters of key transcriptional regulators can be bound by multiple

transcription factors (Brady et al., 2011; Franco-Zorrilla et al., 2014; Taylor-Teeples et al., 2015; Gaudinier et al., 2018). Compared to their targets, upstream transcription factors are also expressed in a higher variety of cell types, allowing them to act pleiotropically. Further support for the pleiotropic effects of transcription factors is given by their ability to have both activating and repressing functions (Brady et al., 2011; Taylor-Teeples et al., 2015). Since the expression of genes in the flavonoid biosynthetic pathway is controlled by transcription factors and products that confer protection against abiotic stressors are also involved in the production of floral color pigments, potential pleiotropic action of these regulators is plausible.

The flavonoid biosynthetic pathway has been well characterized in the South American genus *Petunia* after having undergone recent diversification, approx. 2.85 – 1.3 million years ago (Lorenz-Lemke et al., 2006; Särkinen et al., 2013). This genus comprises species with different pollination syndromes. Two major clades have been identified in this genus: the short tube and long tube clade (Reck-Kortmann et al., 2014). The short tube clade represents the ancestral traits and encompasses the majority of the species, all with very similar phenotypes to attract bee pollinators: purple, UV reflecting flowers with a short tube, producing low amounts of scent (Reck-Kortmann et al., 2014; Amrad et al., 2016). The long tube clade includes three main species, *P. axillaris*, *P. exserta* and *P. secreta*, visited by different pollinator types (Hoballah et al., 2007; Sheehan et al., 2012; Reck-Kortmann et al., 2014). *P. axillaris* displays white, UV absorbing flowers emitting ample amounts of scent volatiles to attract nocturnal hawkmoths (Stehmann et al., 2009; Turchetto et al., 2015; Sheehan et al., 2016) while *P. exserta* has red flowers that are UV reflective and also produce large amounts of diluted nectar, attracting hummingbirds as their primary pollinator (Lorenz-Lemke et al., 2006). *P. secreta* has purple colored, UV reflecting flowers, shown to be primarily visited by solitary bees of the genus *Pseudagapostemon* (Stehmann and Semir, 2005; Rodrigues et al., 2018a, 2018b). All long tube species can be found in the Serra do Sudeste region of southern Brazil.

Based on the most recent phylogeny that robustly separates the short tube and long tube clade and places *P. axillaris* as sister to the two colored and UV reflecting species *P. exserta* and *P. secreta* in the long tube clade (Esfeld et al., 2018) the gain of visible color and loss of UV absorbing pigments occurred from a colorless, UV absorbing ancestor in both species. This notion is also supported by the phylogenetic history of the R2R3-MYB

transcription factors *AN2* and *MYB-FL* (Sheehan et al., 2016; Esfeld et al., 2018). Both transcription factors control the temporal and spatial expression of the *Petunia* flavonoid biosynthetic pathway. The majority of the phenotypic shifts in UV and visible color between the species of the long tube clade have been triggered through changes in these two transcription factors. The gain in visible color of the purple species *P. secreta* has been surprisingly simple: an additional 2 bp deletion in the gene encoding the transcription factor *AN2* has restored the reading frame and allowed *P. secreta* to produce anthocyanin pigments (Esfeld et al., 2018). The evolution of floral color in the red species *P. exserta* has been more complex as it not only entails the regain of visible color but a shift in color from purple to red as well (Berardi et al., 2021). Contrary to the regain of color in *P. secreta*, this has occurred through multiple genetic alterations (Berardi et al., 2021).

While regaining visible color in these two species has been defining for *Petunia* evolution, the loss of UV color that accompanies this, has also been crucial for the emergence of these different pollination syndromes. The *MYB-FL* gene in *P. exserta* contains a 1 bp deletion in the third exon, leading to a truncated protein sequence through the frameshift mutation (Sheehan et al., 2016). In *P. secreta* the *MYB-FL* gene exhibits low expression and possibly encodes a functionally impaired protein or may have reduced transcript stability through a transposon replacing the last five amino acids at the C-terminus (Esfeld et al., 2018). Both types of mutations in *MYB-FL* in *P. exserta* and *P. secreta* lead to a severe reduction in flavonol levels making the flowers appear UV reflective (Esfeld et al., 2018). These findings provide a foundation to help resolve the order in which isolation mechanisms have evolved through phylogenetic and functional analyses.

The phylogenetic relationship of the long tube clade species allows us to estimate what the common ancestor of this clade may have looked like, both in terms of phenotype and genotype. Current estimates describe the common ancestor as most likely being hawkmoth pollinated with white-colored UV-absorbent flowers due to the non-functional *AN2*, down regulated *DPL* and functional *MYB-FL* genes (Esfeld et al., 2018; Sheehan et al., 2016; Berardi et al., 2021). Based on this estimation we can recreate the sequence of evolutionary events that may have caused the shifts in pollination syndromes in the long tube clade. In natural *P. exserta* populations the causal mutation leading to a

non-functional *MYB-FL* gene was found in 138 of 139 genotyped accessions (Sheehan et al., 2016). This points towards a single mutational event having taken place at an early stage during the divergence of the colored species from its non-colored ancestor. Contrary to this, multiple independent loss of function events in the *AN2* gene can be found in *P. exserta* populations as well as a single gain of function mutation in *P. secreta* populations (Esfeld et al., 2018). These evolutionary changes in the *AN2* gene most likely demonstrate that these mutations arose at a later time point during the speciation process of the colored species from a colorless ancestor.

While genetic mutations in the *MYB-FL* gene have most likely led to the produced protein being non-functional, there is still residual, albeit low, *MYB-FL* expression in both *P. exserta* and *P. secreta* as well as residual flavonols produced in the floral limbs of both species (Sheehan et al., 2016; Esfeld et al., 2018). This is comparable with the *LAR1* gene in *Mimulus*, activating flavonol biosynthesis (Yuan et al., 2016). A *cis*-regulatory change in this gene causes differences observed between wildtypes lines but still leads to low levels of *LAR1* transcription (Yuan et al., 2016). This raises the question of whether these mutations in *MYB-FL* found in natural *Petunia* populations really lead to the presumed non-functional protein or if there is still residual activity of the truncated protein causing these observed phenotypes. Assuming possible pleiotropic effects of this transcription factor, such residual activity could be useful for protection against other abiotic stressors. A homolog of the *Petunia MYB-FL* gene, *HaMYB111* in *Helianthus annus* (sunflower) explains most of the variation in UV pigmentation patterns while also co-varying with geoclimatic variables (Todesco et al., 2021). In climates with different relative humidity and temperature values, UV-absorbing pigments can influence desiccation rates (Todesco et al., 2021). As *HaMYB111* is closely related to *MYB-FL*, possible protective effects of UV pigments against other stressors in *Petunia* need to be considered.

Given the complex nature of floral pigmentation and its regulation through transcription factors, the *MYB-FL* gene in *Petunia* offers a compelling system to test the impact of a single gene mutation on the flavonoid biosynthetic pathway, floral UV pigmentation phenotype, pollinator preference and ultimately reproductive isolation of species. Generating a knock-out of the *MYB-FL* gene in *Petunia* would demonstrate whether this gene is highly specific and only responsible for a specific set of functions or whether further possible pleiotropic effects need to be considered as has been found for other

transcription factors (Brady et al., 2011). This could also provide insight into how flavonol and anthocyanin pigments work together to produce both UV and visible color and how this affects pollinator perception.

Testing the effect of a single mutated speciation gene with pollinator behavioral assays is challenging for multiple reasons. Firstly a system is needed where single genes of large effect have been characterized on a molecular level and can be targeted precisely to create a single mutation in an otherwise isogenic background. This allows phenotypic effects resulting solely from this mutation to be analyzed. Further possible pleiotropic effects need to be considered and a wide range of phenotypic traits that may be affected need to be characterized. Ideally, this mutation would only affect a single trait influencing pollinator visitation to eliminate any interaction effects. Lastly the system containing this gene needs to have primary and secondary pollinators present to test the impact of this gene and how it may cause shifts in pollinator preference ultimately affecting reproductive isolation.

Pollinator assays targeting single genes have been performed in various systems, but still include caveats. In *Mimulus* near isogenic lines demonstrated a major shift in pollinator preference based on the *YUP* locus (Bradshaw and Schemske, 2003) but were not able to completely eliminate other confounding factors, such as morphological differences, due to other genes in the introgressed regions. The *Rosea* and *Venosa* genes in *Antirrhinum* were also analyzed in behavioral assays using near isogenic lines and may include such confounding factors as well (Shang et al., 2011). In *Petunia* the impact of the *AN2* gene on pollinator preference was also performed using introgression lines, where the *AN2* chromosome segments were introgressed into a *P. axillaris* background (Dell'Olivo and Kuhlemeier, 2013), but were not able to exclude possible gene interactions of other genes in the introgressed regions. While this should not be an issue for overexpression lines, as were also produced for the *AN2* gene in *P. axillaris* (Hoballah et al., 2007), other confounding factors may influence overexpression results since insertion of the target gene cannot be controlled in the genome.

Previously published analyses on the *MYB-FL* gene demonstrated the importance of UV absorbing pigments for hawkmoth pollinator preference (Sheehan et al., 2016), but used

wild plant species and were thus not able to exclude other confounding factors such as differences in morphology or scent of the compared plant species. Single gene mutations in the *mixta* and *nivea* genes altering floral color appearance have been tested with pollinators (Dyer et al., 2007; Whitney et al., 2009) but these genes have not shown to be speciation genes in *Antirrhinum* species. All of these studies did not yet determine the precise shift in primary as well as secondary pollinator preference when altering a speciation gene responsible for color in an otherwise isogenic background.

Here we present a precise mutation in the speciation gene *MYB-FL* through CRISPR/Cas9 coupled with detailed pairwise pollinator behavioral assays. We show that mutation of this transcription factor leads to few specific alterations in the flavonoid biosynthetic pathway, pointing towards a very specialized function of *MYB-FL*. Altering this single gene causes a major shift in primary pollinator preference, likely affecting reproductive isolation of the species through pollinator selection. These findings demonstrate that speciation occurs through a single mutation of major phenotypic effect, helping settle the debate on whether speciation occurs through gradual change or through single mutational leaps.

Materials and methods

Plant material

P. axillaris ssp. axillaris N (hereafter referred to as *P. axillaris* N) originates from the Rostock Botanical Garden in Germany and is registered in the Amsterdam collection as P. axillaris S26. *P. axillaris ssp. axillaris* P (hereafter referred to as *P. axillaris* P) originates from the University of Bern Botanical Garden (Hoballah et al., 2007). *P. integrifolia ssp. inflata* (*P. inflata* S6, hereafter referred to as *P. inflata*) plants originated from the Vrije Universiteit in Amsterdam and were provided by R. Koes (Hoballah et al., 2007). *P. secreta* was collected in its natural habitat Galpão de Pedra, Rio Grande do Sul, Brazil and maintained by selfing. All plants were grown in commercial soil (70% Klasman substrate, 15% Seramis clay granules, 15% quartz sand) under a light:dark regime of 15:9 h at 22:17 °C in a growth chamber. Plants were fertilized once a week with a nitrogen-phosphorous-potassium and iron fertilizer.

Color and UV image scoring

UV images were recorded with a converted Nikon D7000 SLR camera and Nikon 60 mm 2.8D microlens (aperture F9, ISO 800, shutter speed 1/10 s). The camera was converted to block visible and infrared light, allowing UV light to be recorded using a UV-specific filter (Advanced Camera Services). UV-A light (320-390 nm) was provided as a light source when capturing images through a modified Metz MZ76 flash gun (Advanced Camera Services). Using Photoshop CS4 (Adobe Systems) all images were converted to grayscale. Exposure compensation across the entire image was adjusted depending on the species. Comparison with wildtype *P. axillaris* N or *P. axillaris* P flowers, which are completely absorbent, allowed scoring as either UV reflective or UV absorbent. Color images were recorded with a Canon EOS 60D camera and Canon 35 mm lens (aperture F4.5 – F7.0, ISO 100 – 200, shutter speed 1/30 s). All images were captured under identical incandescent lighting and with a black background for standardized comparison with a color calibration card (X-Rite ColorChecker Classic Mini).

Measurement of additional floral traits

Corolla surface size and morphological traits (stamen and pistil lengths) were analyzed using front and side view photographs. Stamens were numbered according to their placement and size inside the floral tube (1 - 5 ranging from longest stamen to shortest in clockwise order around the floral axis); *Petunia* stamens are arranged in two different sizes (4+1) (Stehmann and Semir, 2005). For morphological traits, flowers were dissected to reveal reproductive organs inside the floral tube. All pictures were analyzed using the ImageJ software to extract lengths and areas of the samples. Three flowers per plant were analyzed as biological replicates. Nectar volume and concentration were measured according to the protocol described in Brandenburg et al., 2012. All flowers were sampled right before the onset of dark in the growth chamber to account for any fluctuations in nectar production during the day:night cycle. Five flowers per plant were analyzed as biological replicates. Methylbenzoate, benzaldehyde and isoeugenol/eugenol compounds were analyzed as described previously in Klahre et al., 2011 and Amrad et al., 2016 using a proton transfer reaction mass spectrometry (PTR-MS) approach. Flowers were collected one day post anthesis right before the onset of dark in the growth chamber (15:9 h light:dark cycle at 22:17 °C). Scent volatiles of all plants were measured for 20 cycles. Five flowers per plant were analyzed as biological replicates. Isoeugenol and

eugenol are isomers and can therefore not be separated via PTR-MS. They were thus analyzed as a single compound. Data was tested for normal distribution and statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463. Results were considered significantly different if $p < 0.05$ and visualized using the ggplot2 package (Wickham, 2016).

Pollen germination measurements

Pollen germination rates were determined after incubation in germination medium. Germination medium consisted of a 10x salt solution (10 mM $CaCl_2$, 10 mM KCl, 8mM $MgSO_4$ x 7 H_2O, 16 mM boric acid, 300 µM $CuSO_4$ x 5 H_2O; salt solution end concentration 1x), 1.5% Casein hydrolysate (end concentration 0.03%), sucrose (end concentration 5%), MES monohydrate (end concentration 15 mM) and PEG 6000 (end concentration 12.5%). The pH value of the medium was adjusted to 5.8 with 1 M KOH and filter sterilized. Single freshly dehisced anthers were dipped into 300 µl germination medium and incubated at room temperature for 2h. Care was taken to not shake samples after incubation to avoid damaging pollen tubes that had grown. Samples were prepared on microscopy slides after incubation and the percentage of germinated pollen grains was determined. Multiple groups were compared via a one-way ANOVA with Tukey *post hoc* comparisons in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463 as data was normally distributed. Results were considered significantly different if $p < 0.05$ and visualized using the ggplot2 package in R (Wickham, 2016).

Identification of flavonoid pigments in petal limbs

To identify the core flavonoid pigments present in petal limbs of *P. axillaris* and mutants, an acid hydrolysis approach was used, after Berardi et al. 2021 with modifications to simplify and accelerate the extraction method due to instability of delphinidin pigments. Petal limbs were harvested, weighed, and quickly soaked in 2N HCl for 15 min in 2 ml screw-cap tubes in the dark. Samples were hydrolyzed (sugars removed to expose the flavonoid aglycones) at 104 °C for 15 min, centrifuged for 1 min at 13000 rpm, and the supernatant removed. The supernatant was centrifuged once more for 1 min at 13000 rpm to remove remaining large particles. An aliquot of 50µl was taken for immediate LC-UV analysis. Due to the low pH of the 2N HCl, extra column washing was incorporated into the HPLC method.

The LC-UV method is described in Berardi et al 2021. Briefly, injections (5 µl) were analyzed with a Dionex Ultimate 3000 Series HPLC with a Phenomenex Kinetix LC C18 column (1.7 µm particle size, 150 × 2.1 mm). Flavonoid aglycones were separated by gradient elution at a flow rate of 0.4 ml/min at 35 °C using solvents A (H_2O with 0.1% formic acid [v/v]) and B (acetonitrile with 0.1% formic acid) with the following protocol: 0–2 min 10% B, 2–7 min 20%, 7–11 min 60% B, 11–12 min 100% B, 12–16 min 100%, 16–16.1 min 10% B and 21 min 10% B. Peaks were detected at 520 nm and 365 nm using a photodiode array detector (DAD) scanning from 190 to 790 nm. Chromatograms were visualized using Dionex Chromeleon (Version 7.1.0.898). Quantification of each compound was conducted by creating a dilution series of a standard compound. Anthocyanidins and flavonols were identified by comparison with retention times and UV spectra of the following: delphinidin, cyanidin, pelargonidin, malvidin, peonidin, petunidin, kaempferol, quercetin and myricetin from Extrasynthese (Genay, France). As data was not normally distributed statistics were calculated with a Mann-Whitney U test for each comparison in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463; data was visualized using the ggplot2 package (Wickham, 2016). Results were considered significantly different if $p < 0.05$.

Spectrophotometric quantifications of floral flavonols and anthocyanidins

Total flavonol and anthocyanidin (anthocyanin aglycone) absorbance was measured for all flower petal limb samples using an Ultraspec 3100 pro (GE Healthcare Life Sciences) spectrophotometer, scanning from 365 nm to 530 nm. Three discs (8 mm diameter) were punched out of the floral limb tissue and placed in 1 ml of 2N HCl to soak for 15 min at room temperature (22 °C), hydrolyzed at 100 °C for 15 min, cooled for 10 min at 4 °C, then centrifuged for 3 min at 14000 rpm. Supernatants were removed and analyzed directly to avoid degradation of pigments. Absorbance values for each sample were measured at 530 nm and 365 nm (Harborne, 1998). Five flowers were sampled per plant to include technical replicates for each biological replicate (individual plant). Results were visualized in R using the ggplot2 package (Wickham, 2016). For comparison of multiple samples statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons after testing data for normal distribution in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463.

Confocal microscopy imaging of epidermal cells

Epidermal peels were taken from the outer rim area of the petal limb of tested species and stained with 0.01% propidium iodide before placing them on microscopy slides for confocal microscopy imaging. Epidermal peels of 3 flowers of each species were taken for comparison. Images were taken with a Leica TCS SP5 microscope. To capture 3D images of the conical cells, we made z-stack images with 0.2 µM z-step size. Images were analyzed using ImageJ and MorphoGraphX.

Generating CRISPR constructs

gRNAs targeting exons 3 and 4 of the *MYB-FL* gene were designed and checked for off-target effects using the CRISPOR tool (Concordet and Haeussler, 2018). Double gRNA constructs under the tomato U6 promoter and *A. thaliana* U6-26 promoter (Genbank X51447.1 and KY080693.1) containing attL sites for Gateway cloning were ordered from Genscript (New Jersey, USA). These constructs then cloned into pDECas9-Kan destination vectors using the LR reaction from the Gateway cloning protocol (Thermo Fisher). Final destination vectors were amplified in *E. coli* and then purified using the extraAxon plasmid mini kit (Axonlab). The destination vectors were then transformed into *A. tumefaciens* strains GV3101 and LBA4404 and grown on solid selective medium at 28 °C for 2 days before being used for subsequent stable plant transformation.

Stable transformation of *P. axillaris*

P. axillaris N and *P. axillaris* P leaves were transformed using *A. tumefaciens* (strains GV3101 or LBA4404) carrying the respective CRISPR construct. *A. tumefaciens* cultures were grown for 2 days on solid media (YEB media with appropriate antibiotics Rifampicin, Streptomycin, Spectinomycin or Gentamycin) at 28 °C before inoculating 5 mL YEB liquid medium (with appropriate antibiotics Streptomycin, Spectinomycin or Gentamycin) with *A. tumefaciens* cultures to grow overnight at 28 °C. Liquid overnight cultures were then centrifuged at 3500 rpm for 15 min, the liquid supernatant discarded and the pellet resuspended in 40 ml of autoclaved MilliQ water with 0.01 mM acetosyringone to aid *A. tumefaciens* infection. Liquid cultures were kept in the dark at room temperature until used for plant inoculation. Leaves from both *P. axillaris* species (from 4-6 week old plants) were sterilized in a 10% hypochlorite solution for 10 min and rinsed five times with autoclaved MilliQ water. The sterilized leaves were then cut into 1

cm^2 pieces and inoculated in the *A. tumefaciens* suspension for 30 min (in the dark with shaking). The leaf fragments were then dried between two layers of sterilized filter paper and transferred to solid Murashige-Skoog (MS) growth medium without antibiotic selection. Leaf pieces were grown in the dark for 7 days at a constant temperature of 24 °C. After this first week, leaf pieces were then transferred to fresh selective growth medium containing Kanamycin to select for the presence of the CRISPR construct. All plates were sealed with medical tape and incubated under the same temperature conditions and a light:dark regime of 16:8 h. Leaf fragments were transferred to fresh media once a week or more often if contaminations were detected for callus growth to be successful. Once shoots started to appear, each shoot was excised from the calli and transferred to MS rooting medium containing kanamycin for further selection of the construct. This allowed roots to form and shoots were transferred to soil (70% Klasman substrate, 15% Seramis clay granules, 15% quartz sand) when their roots reach a length of 2-3 cm. Plants were then grown in the same growth chamber and under the same conditions as all other species (see section plant material for details). Screening for CRISPR mutations was then performed on these plants after one week to allow them to acclimatize to the soil conditions.

DNA extractions and genotyping

Genomic DNA extractions were performed with fresh leaf samples using a modified CTAB (Murray and Thompson, 1980) or SDS protocol. Briefly samples were ground in 200 µl SDS extraction buffer, centrifuged at maximum speed for 10 min. The supernatant was then removed and mixed with 100 µl iso-propanol. After 10 min incubation, samples were centrifuged again at maximum speed for 10 min before the supernatant was discarded. Samples were washed with 100 µl 75% ethanol (centrifuged at maximum speed for 5 min), dried overnight and then resuspended with 100 µl water. Samples were analyzed with a Nanodrop ND-1000 (Thermo Fisher) prior to further analysis. Target sequences were amplified using the GoTaq (Promega) or Q5 polymerase (NEB) with primers depicted in Suppl. Table S3.12. PCR reactions were run according to the manufacturer's protocol with the following parameters: annealing temperature 60 °C, elongation time 1:20 min with 35 cycles. Sequences were obtained through Sanger sequencing (GATC, Cologne, Germany and Microsynth, Balgach, Switzerland) with the primers listed in S3.12. Sequence confirmation for samples of interest were obtained

through cloning (pGEM-T Easy kit, Promega) and subsequent Sanger sequencing. Purification of PCR samples prior to sequencing was done using the NucleoFast 96 PCR plate (Macherey-Nagel). All *MYB-FL* sequences were analyzed manually using the Geneious Primer 2020.2.4 software (Biomatters). Sequences of transformed plants were aligned to existing wildtype sequences of both *P. axillaris* accessions.

RNA extractions, cDNA preparation and quantitative RT-PCR

For each sample type collected, three individual buds (stage 4, 22-30 mm length) from four different plants grown under controlled conditions, were collected (n=12 for each sample type, 4 sample types analyzed), representing biological replicates. The floral limb tissue of the buds was dissected and frozen immediately in liquid nitrogen. Other floral and plant organs were sampled under the same controlled conditions. For leaf samples a single young fresh leaf was sampled while all five sepals of an open flower were pooled for the sepal sample types. All stamens of an individual flower were sampled to analyzed stamens and the pistil of the flower was sampled as a separate sample type. Four to six different plants were grown for sampling and three replicates of every sample type were collected (n=12 for wildtype *P. axillaris* lines, n=18 for *myb-fl P. axillaris* lines). Tissue samples were stored at -80 °C until further processing. RNA extractions were performed using the innuPREP RNA Mini Kit 2.0. All plant tissue was first homogenized in liquid nitrogen before proceeding according to the manufacturer's protocol. Samples were analyzed with a Nanodrop ND-1000 (Thermo Fisher) prior to further analysis. Before proceeding to cDNA synthesis RNA samples were DNase I (Sigma-Aldrich) treated to remove any residual DNA possibly present in the samples. cDNA was synthesized using the qScriber cDNA synthesis kit (Axonlab) and then used for RT-qPCR with the ORA SEE qPCR Green ROX L mix (Axonlab) according to the manufacturer's recommendations.

The expression levels of flavonoid pathway biosynthetic genes (Tornielli et al. 2009) were investigated with an RT-qPCR approach. qPCR primers were used as published previously in Esfeld et al., 2018. Primer sequences for all genes are shown in Suppl. Table S3.19. The reference genes used for all samples were *SAND*, *RAN1* and *ACTIN11* (Mallona et al., 2010). Primer efficiencies for each primer pair were determined using standard curves. RT-qPCR experiments were run on a QuantStudio 5, 384 well Real-Time PCR Machine (Thermo Fisher). All reactions were run in triplicate to calculate the means of

the individual biological replicates using three data points. Using the QuantStudio Design and Analysis (v1.4.3) software the Cq values were determined for calculations. Values of the mutant and wildtype plants of each line were normalized to the three reference genes. For all analyzed biosynthetic genes pairwise comparisons with a Mann-Whitney U test were performed for each sample type of the mean relative expression levels in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463 with results being significantly different if $p < 0.05$. The ggplot2 package was used to visualize the results (Wickham, 2016).

High light stress

4 week old seedlings were subjected to high light stress (750 µmol/m^{2}s intensity) under a light:dark regime of 14:10 at 22:17 °C in a growth chamber during a time period of 4 weeks. Lamps used for illumination cover the full spectrum ranging from UV-A to infrared wavelengths. 30 seedlings were tested per line and chlorophyll measurements were taken using a SPAD-502Plus meter (Konica Minolta). 3 leaves per plantlet were measured and the average calculated to determine the mean chlorophyll content. As the data was not normally distributed pairwise comparisons were performed with a Mann-Whitney U test in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463; data was visualized using the ggplot2 package (Wickham, 2016). Results were considered significantly different if $p < 0.05$.

RNA sequencing

The same samples (n=16) used for RT-qPCR analysis were used for RNA sequencing to obtain a specific overview of up- or downregulation patterns not only in genes of the flavonoid biosynthetic pathway but also in genes involved in other processes. The quantity and quality of the extracted RNA was assessed using a Thermo Fisher Scientific Qubit 4.0 fluorometer with the Qubit RNA BR Assay Kit (Thermo Fisher Scientific) and an Advanced Analytical Fragment Analyzer System using a Fragment Analyzer RNA Kit (Agilent), respectively. Thereafter cDNA libraries were generated using an Illumina TruSeq Stranded mRNA Library Kit (Illumina) in combination with IDT for Illumina IDT for Illumina – TruSeq RNA UD Indexes (Illumina). The Illumina protocol was followed with the recommended input of 1000 ng total RNA. The quantity and quality of the generated NGS libraries were evaluated using a Thermo Fisher Scientific Qubit 4.0 fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and an

Advanced Analytical Fragment Analyzer System using a Fragment Analyzer NGS Fragment Kit (Agilent), respectively. The pooled libraries were 100 bp single end sequenced using a NovaSeq 6000 SP Reagent Kit, v1, 100 cycles (Illumina) on an Illumina NovaSeq 6000 instrument. The quality of the sequencing runs was assessed using Illumina Sequencing Analysis Viewer (Illumina version 2.4.7) and all base call files were demultiplexed and converted into FASTQ files using Illumina bcl2fastq conversion software v2.20. The average number of reads/ library was 41 million. The RNA quality-control assessments, generation of libraries and sequencing run were performed at the Next Generation Sequencing Platform, University of Bern, Switzerland.

Read processing and differential expression analysis

The quality of the Illumina read data was processed using Trimmomatic v.0.36 (Bolger et al., 2014). This removed Illumina adaptor sequences and trimmed low quality regions. Processed reads were then mapped to the reference genome of *P. axillaris* N v.4.0.2 using STAR v.2.6.0c (Dobin et al., 2013) in two-pass mode, with splice junctions –sjbdOverhang 100. Reads that mapped > 20 times were discarded. Reads were counted per gene using featureCounts v1.5.2 (Liao et al., 2014). Differential expression analysis was performed between mutant *P. axillaris* N *myb-fl* and *P. axillaris* N lines, and was performed with DESeq2 v.1.26.0 (Love et al., 2014) in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463. The *myb-fl* mutant samples of each line were compared to the wildtype *P. axillaris* N of the same line. Read counts were normalized with rlog-transformation in DESeq2 and sample replicates were used to compute mean counts per gene. Genes were considered significantly differentially expressed between the two lines if expression difference was at least 2-fold and p-value < 0.05. Functional annotations of differentially expressed genes were determined using the functional annotation scripts in Maker (v2.31.9) to call blastp (uniprot database accessed on 09/03/2020) and interproscan (v5.33.72.0). Interproscan additionally adds PFAM hits and assigns GO (gene ontology) terms.

Behavioral experiments

B. terrestris (bumblebee) colonies were obtained from Andermatt Biocontrol AG (Bombus Maxi; Grossdietwil, Switzerland) and maintained in the dark before experimentation (at 21 °C). Bumblebees were fed with pollen three times a week and were allowed to feed on a provided sugar solution (Biogluc, Biobest) at all times. The day

before being used for behavioral assays, single bumblebees were extracted from the colony, given 500 µl of a 20% sucrose solution and isolated overnight in the dark. Assays were performed in a scent saturated greenhouse during sunny conditions (greenhouse temperature 25 °C) in a flight cage with the following dimensions: 250 x 130 x 150 cm. Each bumblebee was presented with an array containing two plant species for choice experiments. Each array contained four plants per species, which were arranged in a 4 x 2 pattern with 30 cm distance between each plant. To control for differences in floral display, plants were manipulated to display the same number of flowers per plant (1 flower per plant for long tube species, 2 flowers per plant for short tube species). All flowers used for experiments were second day flowers post anthesis. Naive bumblebees were allowed to fly for 10 min each and all choices were recorded. Encounters were classified into three groups: touch, visit and forage (Fig. 3.1 A). A touch described the shortest type of encounter where the bumblebee made contact with the flower briefly but failed to land on the flower. A visit was recorded if a bumblebee landed on the flower for 1-2 seconds but did not forage for pollen or nectar. Encounters were categorized as forages if the bumblebee landed on the flower for 3 or more seconds and actively foraged for pollen or nectar. If a bumblebee failed to fly or visit a flower during this time period, it was removed and not used for any further experiments (Suppl. Table S3.18). All bumblebees were only used for a single choice assay.

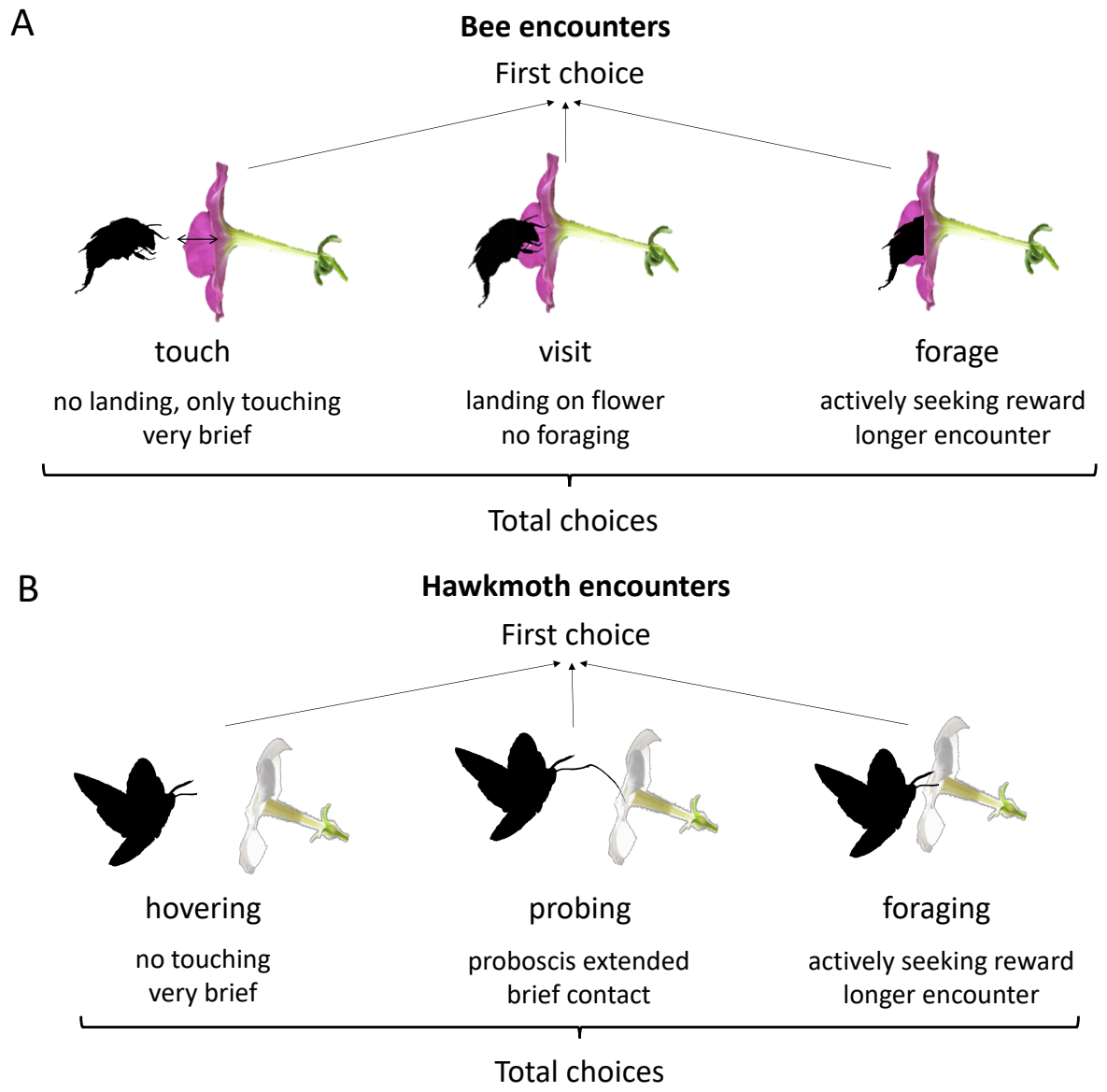

Figure 3.1: Recording pollinator encounters during 10 min trials. A single pollinator's movement was tracked for a duration of 10 min and each encounter with a flower was classified into one of three behavioral categories (touch, visit or forage for bees and hovering, probing or foraging for hawkmoths). All encounters combined together resulted in the total number of choices to a plant, while the very first encounter was recorded as the first choice. A) Bee encounters were classified into three categories: touch, visit or forage. A touch describes the shortest type of encounter, where bees do not land on the flower but merely touch the floral limb. Visits were classified as such if bees landed on the flower but did not forage for pollen or nectar. Forages were the longest types of encounters, where bees actively foraged for rewards. (B) Hawkmoth encounters were classified into three categories: hovering, probing and foraging. If a hawkmoth hovered in front of an open flower but did not extend its proboscis it was classified in this category. Probing was recorded if a hawkmoth probed the flower with its proboscis but did not access the reward. Foraging describes the longest type of encounter where hawkmoths extended their proboscis into the tube of the flower and actively foraged for nectar.

The same experimental setup was also performed for a second type of bee pollinator: solitary bees *Osmia cornuta*. Pupae of *O. cornuta* were obtained from Naturschutzcenter (Rottenburg, Germany) in December and kept at 4° C until they were removed in spring prior to eclosion. Solitary bees were fed with pollen three times a week and were allowed to feed on a provided sugar solution (Biogluc, Biobest) at all times. Solitary bees were used for experiments 2-5 days after emerging from the pupae and isolated overnight in the dark the day before experiments. Assays were performed as described for bumblebees in a flight cage with the following dimensions: 180 cm x 60 cm x 60 cm. Each array contained 2 plants per species, arranged in a 2 x 2 pattern with 20 cm distance between the plants. As half the number of plants were used, compared to the bumblebee setup, we controlled for floral display by allowing twice the number of flowers per plant (2 flowers per plant for long tube species, 4 flowers per plant for short tube species; flowers second day post anthesis). Recording experiments was done as described for bumblebees.

M. sexta female pupae were obtained from the group of Prof. Monika Stengl at the University of Kassel (Kassel, Germany). Pupae were reared in a climate chamber under controlled temperature and humidity with a light:dark regime of 16:8 h. After eclosion adult moths were fed with a nectar solution (protocol kindly provided by André Arand, Kassel, Germany) for two days. Nectar solution consisted of glucose monohydrate (33.72 g), fructose (27.68 g), sucrose (158.94 g), vitamin B1 (0.008 g), vitamin B2 (0.0036 g), vitamin B6 (0.0036 g), folic acid (0.0036 g) and biotin (0.00032 g) dissolved in 1L of water. Behavioral assays were conducted in a dark room with controlled temperature and humidity (21 °C, 60%) in a flight cage of 295 x 205 x 160 cm. Plants and moth growth chambers were synchronized to the same light:dark cycle to best mimic natural situations. Moths were transferred to the flight cage room 30 min prior to the start of experiments to acclimatize and initiate wing fanning. Low intensity lights emitting UV-A light as well as light in the visible blue range (spectral distribution 350 – 500 nm, average intensity 1 lux) was used during experiments to mimic lighting conditions during *M. sexta* natural flying conditions. Moonlight intensity has been calculated to be between 0.5 and 2 lux depending on the moon phase and lighting conditions (Bruce-White and Shardlow, 2011; Weaver, 2011; Yorzinski et al., 2015). Each moth was presented with an array containing two plant species for choice experiments. Each array contained four plants per species,

which were arranged in a 4 x 2 pattern with 30 cm distance between each plant. To control for differences in floral display, plants were manipulated to display the same number of flowers per plant (1 flower per plant for long tube species, 2 flowers per plant for short tube species). Naïve moths were observed for 10 min each. Due to the foraging behavior of moths being different to that of bumblebees, encounters were classified into three categories different to those of the bumblebees: hovering, probing and foraging (Fig. 3.1 B). An encounter was categorized as hovering if the moth actively displayed interest in the flower and hovered in front of it but did not touch it. Moths probed on the flowers if they extended their proboscis to touch the flower but did not forage for nectar. Encounters were classified as foraging if moths extended their proboscis to the base of the floral tube and foraged for nectar. All moths were used only once for a choice assay to eliminate any bias due to learning behavior.

Significant differences for all behavioral assays with pairwise comparisons of flower species were performed in R v3.6.1 (R Core Team, 2017) using an exact binomial test and RStudio v1.3.463. Results were considered significantly different if $p < 0.05$. Data was visualized using the ggplot2 package (Wickham, 2016).

Results

CRISPR/Cas9-mediated knockout of *MYB-FL* gene causes a shift in the balance between UV and visible color

We aimed to knock out the *MYB-FL* gene in *P. axillaris* with UV absorbent flowers to produce UV reflective flowers. To perform the knockout, we used a CRISPR/Cas9 approach using two gRNAs to target the third and fourth exons in *P. axillaris* N and P accessions (Fig. 3.2 A). Two independent mutations were generated in each line. A 1 bp insertion and 5 bp deletion both in exon 4 were produced in *P. axillaris* P, while a 2 bp insertion in exon 3 and 1 bp insertion in exon 4 were found in *P. axillaris* N (Fig. 3.2 A). The mutations in each line led to an early stop codon, resulting in a truncated and presumably non-functional *MYB-FL* protein (Fig 3.2 A). Mutations in exon 3 led to a truncated R3 domain while the early mutations in exon 4 removed large portions of the C-terminus of the protein. All mutated lines showed a shift in floral UV phenotype; flowers were no longer UV absorbent but appeared UV reflective (Fig. 3.2 B). Additionally,

we observed a shift in visible color phenotype. Interestingly this shift in color was not the same for both *P. axillaris* lines. Instead of white, mutant *P. axillaris* N lines appeared light pink and *P. axillaris* P lines appeared faint pink (Fig. 3.2 B). Mutating *MYB-FL* therefore seems to cause a major shift in UV color but also a slight shift in visible color.

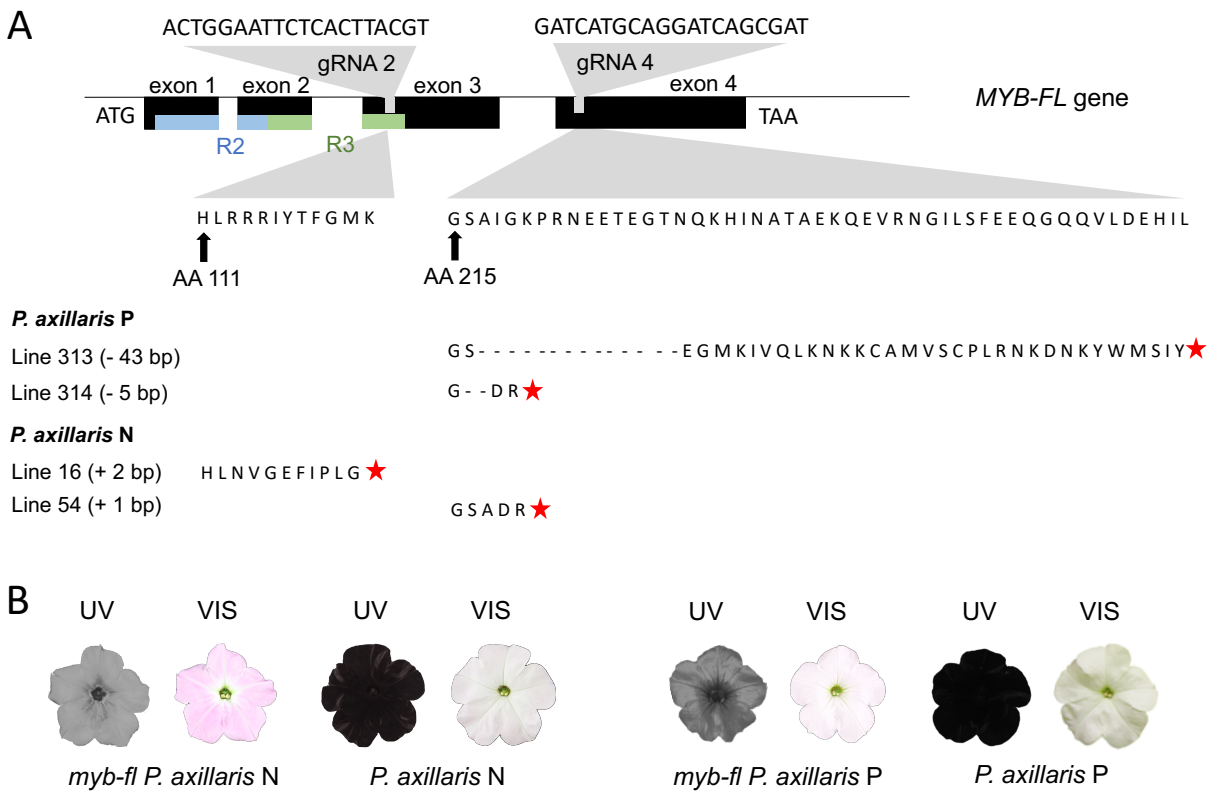

Figure 3.2: Targeting the *MYB-FL* gene via CRISPR/Cas9. (A) A double gRNA CRISPR/Cas9 construct was used to target exon 3 and 4 of the *MYB-FL* gene in *P. axillaris* N and P. Two independent mutant lines of each species were generated through different insertions and deletions all leading to a truncated protein through a premature stop codon. (B) The mutations in the *MYB-FL* gene led to altered UV and visible color phenotypes. Flowers no longer appeared absorbent under UV light but reflective for both *P. axillaris* species. *P. axillaris* N mutants showed a light pink visible coloration while the *P. axillaris* P mutants were only faintly pink colored.

To characterize the biochemical aspects of the *MYB-FL* knockout on floral color, we analyzed the flavonoid pigment profile of the *P. axillaris myb-fl* mutants. We first used an HPLC approach to determine the exact composition of different flavonoid aglycones using acid hydrolysis, which removes flavonoid decorations for easier identification of the underlying pigment (Harborne, 1998; Berardi et al., 2021). Compared to wildtype plants, we detected low amounts of the flavonol quercetin in the mutants, as well as slightly

elevated levels of the anthocyanidins (anthocyanin aglycone) delphinidin and cyanidin (Fig. 3.3 A, all p-values of statistics in Suppl. Table S3.10). Quercetin was the dominant flavonol in both wildtype and mutant lines (96.4 % WT, 98.1 % mutant line), and delphinidin the dominant anthocyanidin (100% WT, 87.9 % mutant line) but total concentration of these compounds varied between the mutants and wildtypes (Fig. 3.3 A). Since the analysis of composition of hydrolyzed flavonoids through the highly specific HPLC method demonstrated that the composition of compounds was fairly simple, we used UV-VIS spectrophotometry for general analysis of total flavonols and anthocyanidins. Flavonol absorbance was significantly reduced in the *myb-fl* mutants compared to the wildtype line, while anthocyanidin absorbance was significantly higher in the *myb-fl* mutants (Fig 3.3 B, detailed statistics in Suppl. Table S3.10). We observed no differences in flavonol absorbance between the *myb-fl* mutants of *P. axillaris* N and P, but *P. axillaris* N *myb-fl* mutants had a higher anthocyanidin absorbance than *P. axillaris* P *myb-fl* mutants (Fig. 3.3 B). Overall, *myb-fl* lines showed a reduction in flavonols responsible for UV color as well as an elevation in anthocyanidin pigments responsible for visible color, possibly through a trade-off of the anthocyanin and flavonol branches of the flavonoid biosynthesis pathway as previously observed in a different *P. axillaris* background (Sheehan et al., 2016).

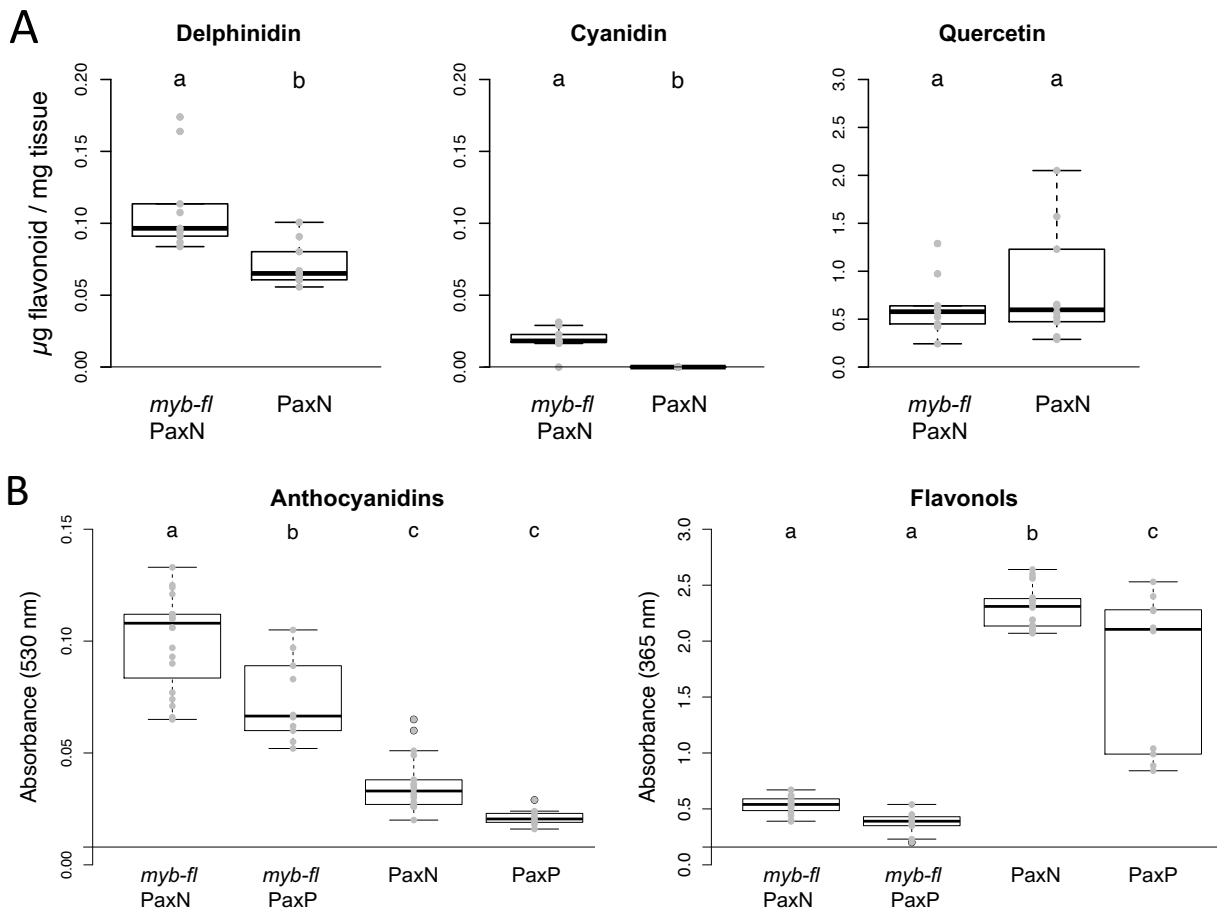

Figure 3.3: Analysis of the floral pigments in *myb-fl* mutants. (A) The *myb-fl* mutants produce two classes of anthocyanidin pigments (delphinidin and cyanidin) and one flavonol pigment (quercetin) in the *P. axillaris* N lines. Boxplots represent mean values of 8 replicates per line. Statistics were calculated using a Mann-Whitney U test. Letters indicate statistically significant differences (full statistics in Suppl. Table S3.10). (B) Total flavonol and anthocyanidin absorbance values of the wildtype and mutant lines as determined through spectrophotometric analysis. Letters indicate statistically significant groups calculated using a one-way ANOVA with Tukey *post hoc* comparisons (full statistics in Suppl. Table S3.10).

myb-fl mutation does not alter other phenotypic traits

To determine whether the mutation of *MYB-FL* only specifically altered the color phenotype we were aiming to change, we tested various other phenotypic traits that could potentially influence pollinator preference. None of the other measured pollination syndrome phenotypic traits (corolla size, pistil and stamen length, nectar volume and concentration, pollen germination rate) showed differences between wildtype and their respective mutant lines (Fig. 3.4 A-E). The only differences that were observed were between the different *P. axillaris* lines, as can be seen for nectar concentration and volume (Fig. 3.4 C-D). All p-values of calculated statistics can be found in Suppl. Tables S3.3 - S3.6.

Not only is color of a flower and its perception influenced by the composition of flavonols and anthocyanins, but also the shape of the epidermal cells that contain the vacuoles carrying these pigments. We visualized the shape of the conical epidermal cells of different *Petunia* species using propidium iodide (PI) stained samples for confocal microscopy. All four tested wild species (*P. inflata, P. secreta, P. axillaris* N and P) showed varying shapes of conical cells (Fig. 3.4 I). We detected no differences in cell shape between the *myb-fl* mutants and their corresponding wildtype lines. The mutation in *myb-fl* therefore does not affect other phenotypic parameters that may alter floral color, showing that the differences in visible and UV color phenotype are solely due to differences in flavonoid pigment composition. We also looked at potential differences in scent production between the *myb-fl* mutant lines and their respective wildtype counterparts. As for previous analyzed parameters, no differences were detected for scent production (methylbenzoate, benzaldehyde, isoeugenol/eugenol) between the *myb-fl* mutants and wildtype lines (Fig. 3.4 F-H). However, there are differences in benzaldehyde production that can be found between the different *P. axillaris* lines (Fig. 3.4 F-H). Overall, through the CRISPR/Cas9 approach we were able to demonstrate that mutating a single gene, *MYB-FL*, alters only floral color eliminating any possible confounding effects due to further phenotypic mutations.

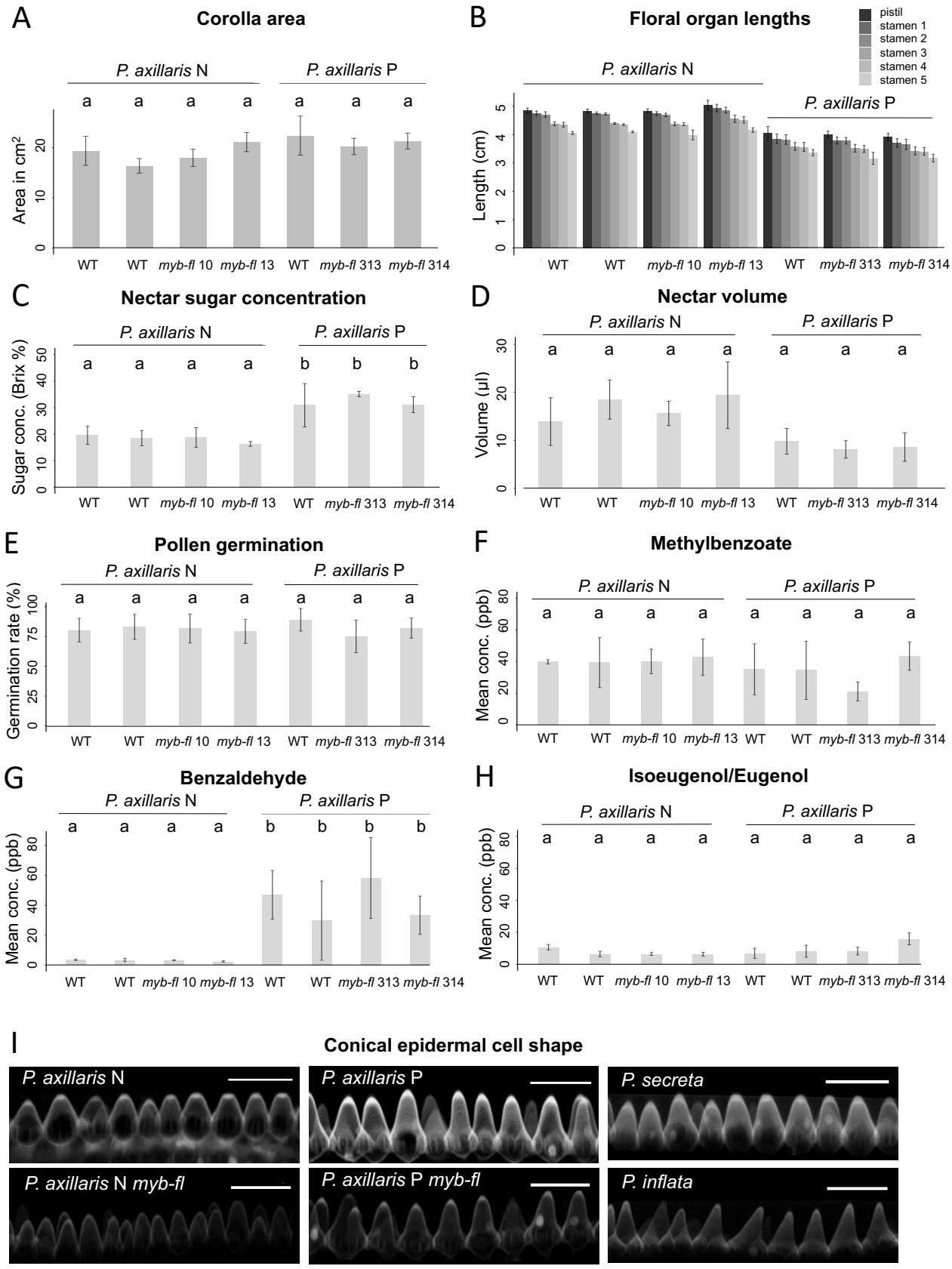

Figure 3.4: Phenotypic measurements of *myb-fl* mutant lines compared to wildtype *P. axillaris*. All bars depict mean values ± SD. Statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons and can be found in Suppl. Tables S3.3 - S3.6. (A) Corolla areas for mutant and wildtype lines.

(B) Pistil and stamen lengths were measured. All five stamens were measured individually. (C) Sugar concentration and (D) volume of nectar in the different samples was analyzed. (E) Pollen germination rates 2h post inoculation in germination medium were determined. (F) Scent volatiles methylbenzoate, (G) benzaldehyde and (H) isoeugenol/eugenol were analyzed for the mutant and wildtype lines via PTR-MS. (I) Conical epidermal cell shapes of four natural *Petunia* species as well as two *myb-fl* mutant lines. Images were acquired using confocal microscopy with PI stained samples. Scale bars represent 50 µM in length.

myb-fl **mutation leads to specific changes in expression of few genes of the flavonoid biosynthetic pathway**

Given the observed changes in both UV and visible floral phenotypes, we investigated whether the mutation in *MYB-FL* affected the expression of downstream flavonoid biosynthetic genes as well as its own expression possibly by inducing autoregulation. We predicted that *MYB-FL* target genes *HT1/F3'H* and *FLS*, key biosynthetic genes in flavonol biosynthesis, would be down-regulated (Sheehan et al. 2016), and anthocyanin biosynthetic genes possibly up-regulated. An RNAseq experiment was conducted with two lines of *P. axillaris* N *myb-fl* mutants. The mutation of *MYB-FL* led to a truncated protein and a 1.38x lower expression of *MYB-FL* itself in the mutants (Fig. 3.5), possibly through the process of nonsense mediated decay. Additionally, the observed truncated protein of *MYB-FL* in *myb-fl* mutant lines altered the expression of downstream genes in the flavonoid pathway. A strong decrease in flavonol biosynthetic genes (*HT1/F3'H* and *FLS*) was detected, but only modest changes in anthocyanin related genes (upregulation of *ANS*, *HF2/F3'5'H*, *MF1/3'5'AMT* and *MF2/3'5'AMT*) were observed (Fig. 3.5, for full statistics see Suppl. Table S3.1). This is the first time we have been able to demonstrate that *HT1/F3'H* is under direct control of *MYB-FL*. Flavonoid biosynthetic gene expression was quantified with RT-qPCR, supporting the patterns observed in the RNAseq experiment for the flavonol biosynthetic genes (Suppl. Fig. S3.1, full statistics in Suppl. Table S3.7); *HT1/F3'H*, *FLS* and *MYB-FL* expression was reduced in *myb-fl* mutant lines, while no upregulation of anthocyanin related genes was detected. These few changes in the expression of genes in the flavonoid biosynthetic pathway, point towards *MYB-FL* being very specific in its function.

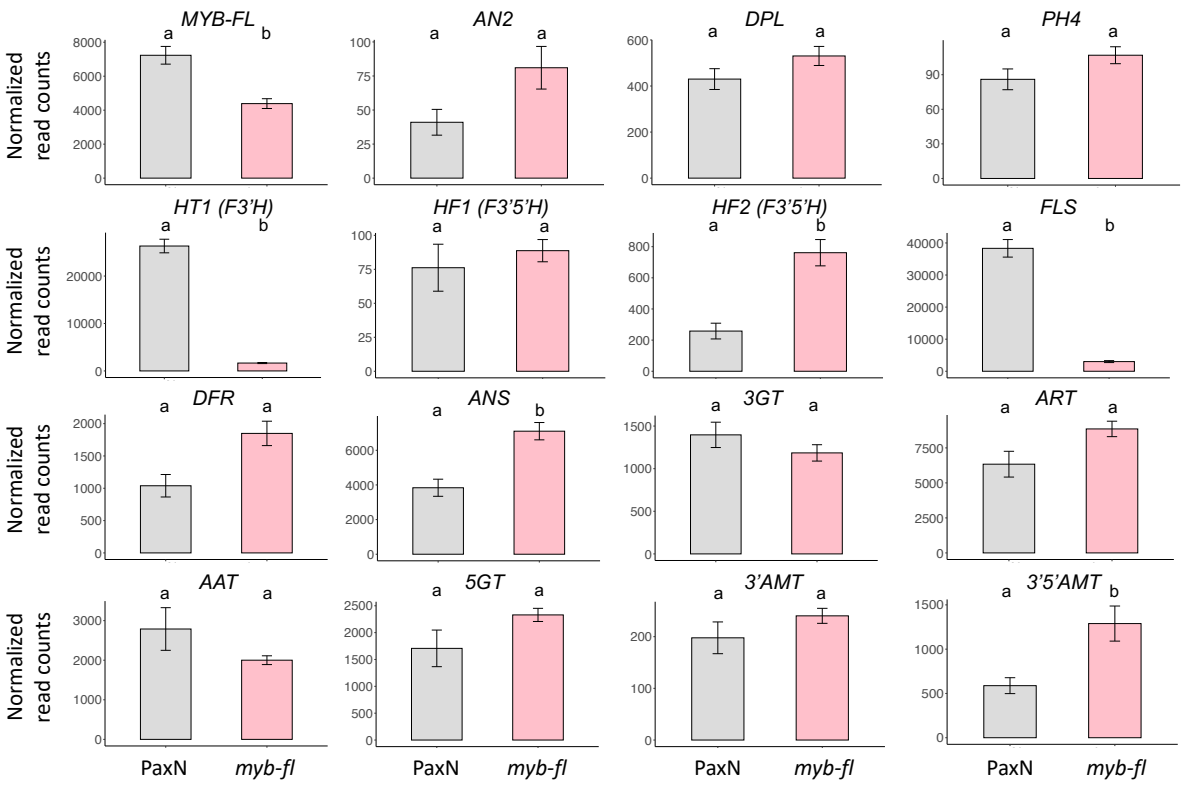

Figure 3.5: **Expression of few genes in flavonoid biosynthetic pathway is altered through *MYB-FL* mutation**. Gene expression was measured in *P. axillaris* N *myb-fl* mutants and wildtype plants. Differential expression analysis was performed between mutant *P. axillaris N myb-fl* and *P. axillaris* N lines and read counts were normalized with rlog-transformation (full statistics in Suppl. Table S3.1). Bars show means of mapped reads for 8 biological replicates. Error bars represent ± SE. Letters depict significant differences (p-value < 0.05).

Global DE analysis indicates a role of *MYB-FL* in defense responses

To consider other aspects of plant development besides the flavonoid biosynthetic pathway, we investigated whether there were any global gene expression changes in petal limb tissue in *myb-fl* mutants with a differential expression analysis. Only 55 genes were significantly differentially expressed (DE), using a filter of a log 2-fold change value greater than 1 or less than -1 corresponding to a 2-fold change in expression, and a p-value < 0.05 (Suppl. Table S3.2). Since flavonols accumulate in plant tissues under high light (Tattini et al., 2005; Agati and Tattini, 2010; Liu et al., 2018), *P. axillaris* grows in high light environments (Turchetto et al., 2014, 2019) and DE genes were potentially involved in light induced stress responses (e.g. DNA photolyase Peaxi162Scf00034g01311 or Cryptochrome/DNA photolyase Peaxi162Scf00213g01024; see Suppl. Table S3.2), we investigated whether a phenotypic difference between mutant and wildtype lines could be observed under high light stress. Plants were tested in climate

chambers with full light spectrum lamps, including UV-A wavelengths. No differences in chlorophyll concentration of leaves were observed between the mutants and wildtypes for both independent *myb-fl* mutant lines we tested (Fig. 3.6, statistics in Suppl. Table S3.9). We additionally did not observe any fitness-related differences in number of flowers produced or flowering time. Thus, the *myb-fl* mutation appears to influence the expression of only very few target genes in the genome and does not alter the response of the plant to abiotic stressors, such as light intensity.

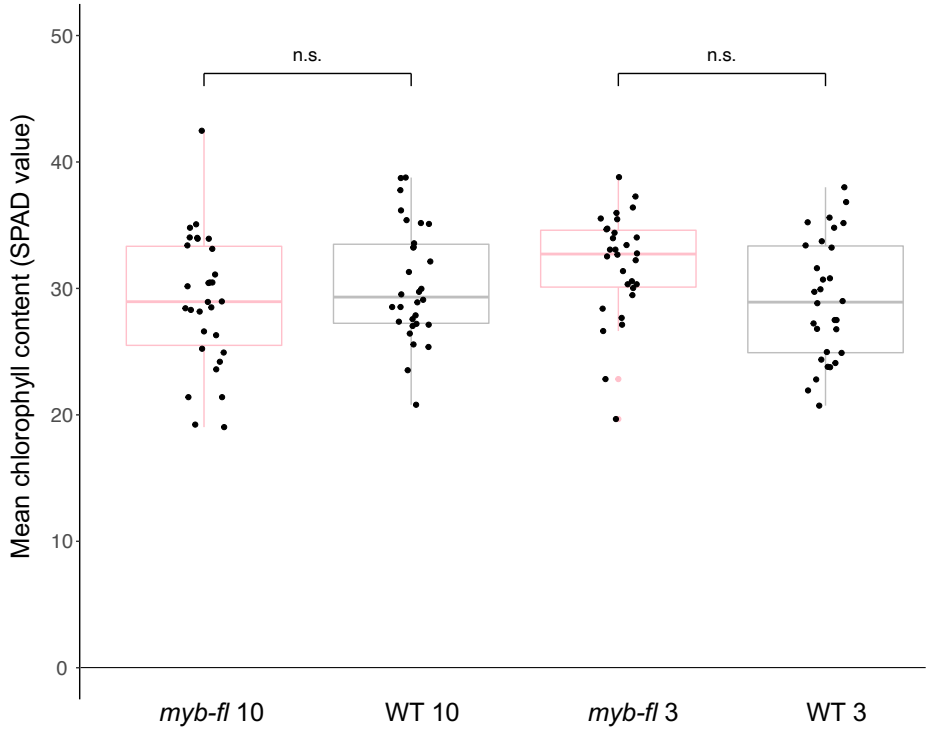

Figure 3.6: **Chlorophyll content (SPAD values) after high light treatment**. Both *myb-fl* mutant lines and their wildtype controls were analyzed. Boxplots represent mean values for 30 replicates of each line (mean value of each replicate determined by measuring 3 leaves). Statistics were calculated using a Mann Whitney U test and no significant differences were detected (full statistics given in Suppl. Table S3.9).

Transcription factor specificity achieved through spatial expression

To determine if the specificity of *MYB-FL* is achieved through spatial expression, we tested its expression levels via RT-qPCR in floral and plant organs (stage 4 bud limbs, pistils, stamens, sepals and leaves). Levels of *MYB-FL* expression were low in all tested organ types except floral limbs, where the mutant lines showed significantly higher expression levels compared to the mutant lines (Fig. 3.7, detailed statistics in Suppl. Table

S3.8). Pistils also showed significantly higher *MYB-FL* expression in the wildtype plants, but overall flavonol levels were much lower than compared to floral limbs (Fig. 3.7). No differences were found between the *P. axillaris myb-fl* mutants and wildtype lines in the other organs (stamens, sepals, leaves). As a comparison the *AN2* gene, responsible for activation of visible pigment production, was also tested in all organs. No significant differences between expression levels of mutant and wildtype lines were found for this gene (Fig. 3.7). Expression levels were the highest in the floral limbs and lower in all other tested organ types. Overall the specific expression of *MYB-FL* in floral limbs supports the hypothesis that *MYB-FL* is only expressed in certain organs. The specificity of the transcription factor *MYB-FL* is therefore likely to be caused through a precise regulation of its spatial expression but could additionally be controlled through differences in temporal expression as well.

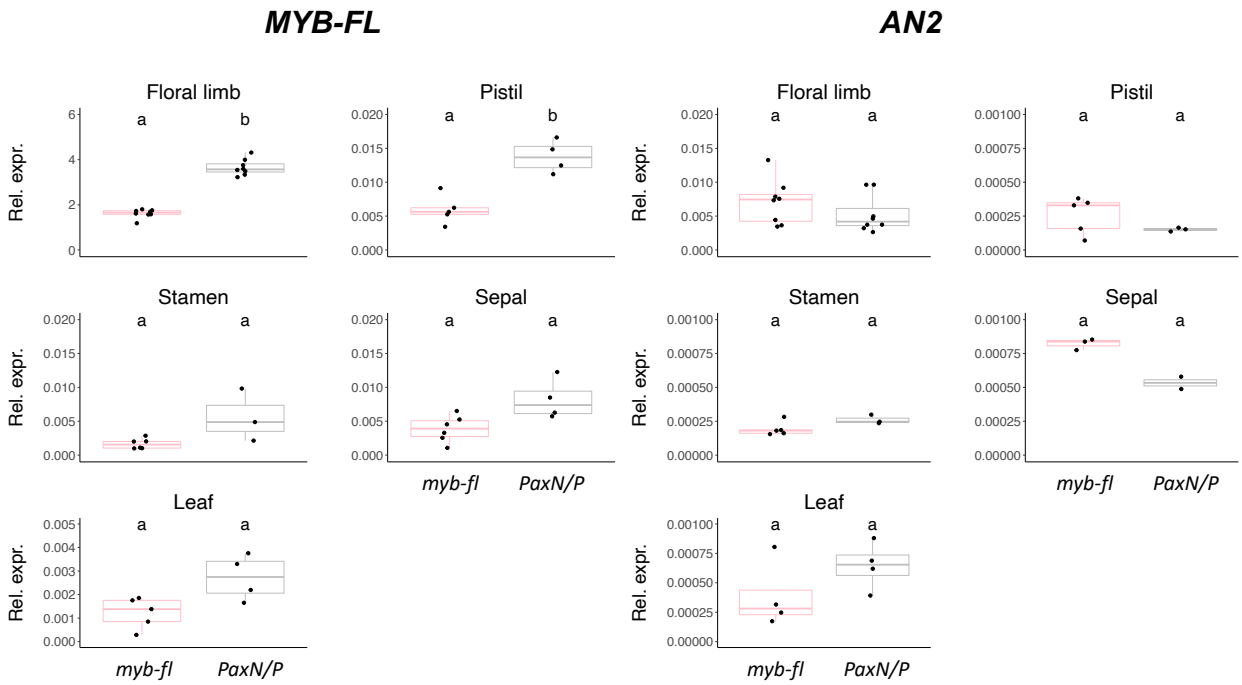

Figure 3.7: **Gene expression levels determined by RT-qPCR of the *MYB-FL* and *AN2* transcription factors**. Gene expression was quantified in *myb-fl* mutant and wildtype lines of both *P. axillaris* N and P (pink = *myb-fl*, gray = *P. axillaris*). Expression is shown relative to the expression of the three housekeeping genes *SAND, RAN1* and *ACTIN11*. Statistics were calculated using a Mann Whitney U test and letters depict significant differences (full statistics shown in Suppl. Table S3.8).

Primary pollinator preference is distinct in natural *Petunia* species

To assess the effect of a single trait on pollinator attraction in *Petunia*, establishing the precise preference of different pollinators for *Petunia* species is crucial. In nature hawkmoths have been observed visiting *P. axillaris* at night, while bee visits have also been observed during the day to these flowers (Hoballah et al., 2007). *P. inflata* on the other hand is only visited during the day, mainly by bees (Hoballah et al., 2007). Pollination in *Petunia* species is therefore dominated by a single pollinator species, but not exclusively pollinated by only one pollinator type. When compared directly hawkmoths (*M. sexta*) prefer visiting *P. axillaris* over *P. inflata* while the opposite has been shown for bumblebees (*B. terrestris*) (Hoballah et al., 2007). *P. secreta* is pollinated by halictid bees in the wild (Rodrigues et al., 2018b), and is closely related to the hawkmoth pollinated species *P. axillaris*. However, *P. secreta* has not yet been used for comparative behavioral assays. Compared to the short tube bee pollinated species *P. inflata*, *P. secreta* has a lower amount of anthocyanidins and a higher amount of flavonols (Esfeld et al., 2018), which may affect its appearance to pollinators. Also, phylogenetically *P. secreta* represents a reversal of bee pollination syndrome from a hawkmoth syndrome and is morphologically not completely adapted to bee pollinators. Due to its long, narrow floral tube, bee pollinators have difficulties landing on the limb and cannot access any nectar, only allowing them to collect pollen (Rodrigues et al., 2018b). Will these differences between the bee pollinated species in *Petunia* affect pollinator behavior?

To assess the preference of pollinators for different wild *Petunia* species, we set up pairwise comparisons of the three wildtype *Petunia* species (*P. inflata*, *P. secreta*, *P. axillaris*) using three different pollinator types (n=20 for each comparison): model pollinators hawkmoth *M. sexta*, bumblebee *B. terrestris*, as well as a solitary bee *O. cornuta*, which is closer in size to the natural pollinator of *P. secreta* than the model organism *B. terrestris*. Behavioral measurements, such as first choice (noting the first plant visited at the start of any array) and total choices (all plant choices recorded during the 10 min duration of the experiments) were recorded (see Fig. 3.1). Similar to Hoballah et al., 2007, hawkmoths preferred *P. axillaris* over *P. inflata* and bumblebees preferred *P. inflata* over *P. axillaris* for both first and total choices (Fig. 3.8). Solitary bees also preferred *P. inflata* over *P. axillaris* and were even more selective towards the colored species than bumblebees as not a single solitary bee chose *P. axillaris* for any of their choices (Fig. 3.8).

When using *P. secreta* instead of *P. inflata* in these experiments, allowing the pollinators to choose between *P. axillaris* and *P. secreta,* hawkmoths significantly preferred *P. axillaris* for first and total choices while bumblebees only significantly preferred *P. secreta* for total choices. The comparison of *P. axillaris* with *P. inflata* invokes a more selective bee response than when using *P. secreta*. Interestingly the results were not the same for solitary bees and bumblebees, demonstrating potential differences not only between pollinator groups but also between species in a specific group.

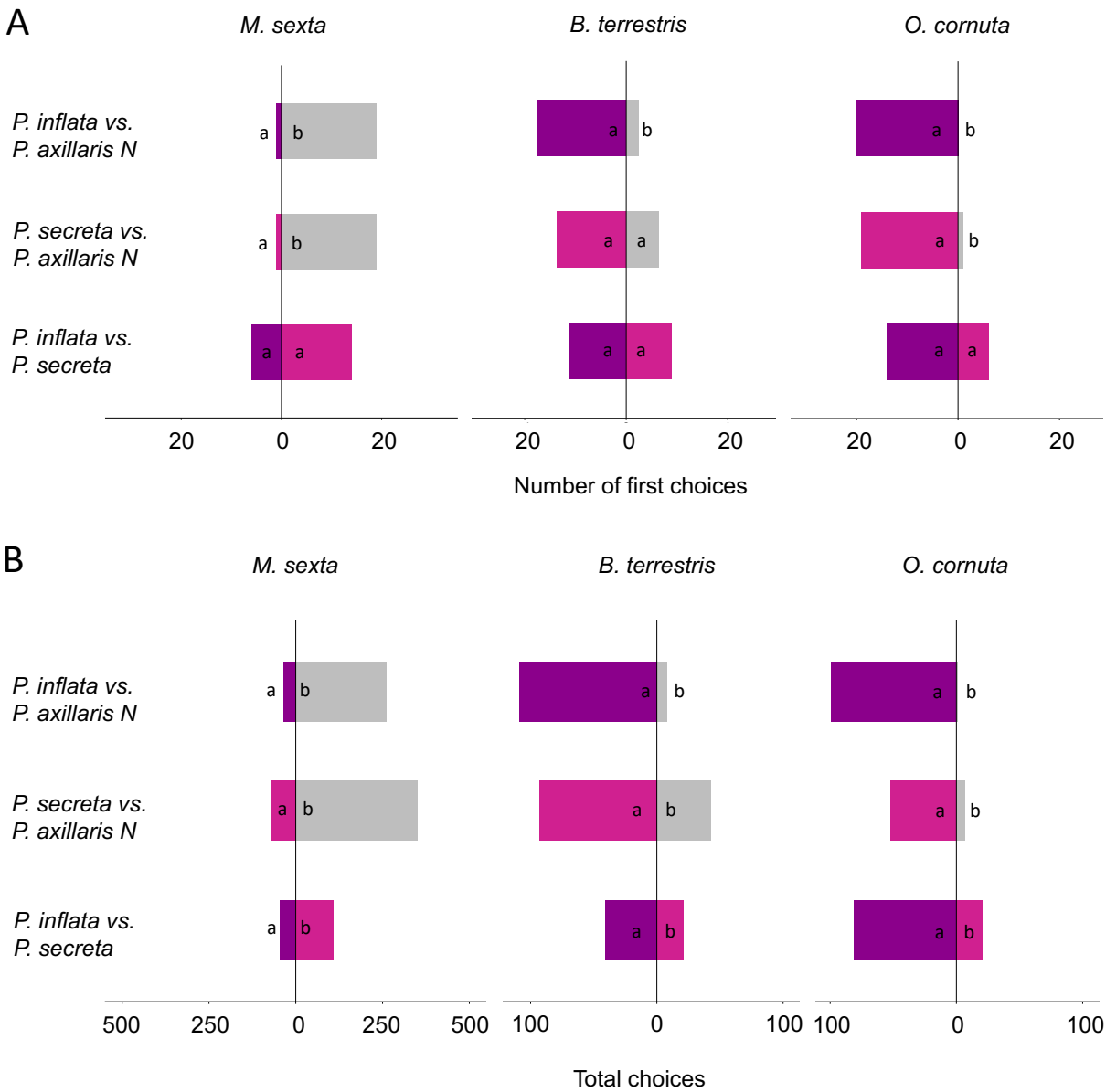

Figure 3.8: Pairwise comparisons of wildtype *Petunia* species with hawkmoth and bee pollinators (*M. sexta*, *B. terrestris* and *O. cornuta*). Plant species labels on the left side of the graphs show the different pairwise comparisons (purple = *P. inflata*, pink = *P. secreta*, gray = *P. axillaris* N). Statistical

differences between groups were calculated using an exact binomial test and are depicted by letters. Full statistics are represented in Suppl. Tables S3.12 (*M. sexta*), S3.14 (*B. terrestris*) and S3.16 (*O. cornuta*). (A) Bars demonstrate the total number of pollinator first choices for pairwise comparisons of wildtype *Petunia* species (n=20 pollinators for each comparison, total n=180). Hawkmoths clearly preferred the white-colored and UV absorbent *P. axillaris* species over the colored species, while the opposite was visible for bees. (B) Total choices of pairwise comparisons of wildtype *Petunia* species (n=20 pollinators per comparison, total n=180). Each pollinator was allowed to make multiple choices during a 10 min time period and bars display the total amount of choices for each 10 min experiment.

Using *P. secreta* in behavioral assays also allowed us to perform control experiments with two UV reflective species from different clades within the *Petunia* genus (*P. inflata* and *P. secreta*). Although both UV reflective, these species may not appear identical to pollinators due to their differences in flavonoid concentrations: *P. secreta* has a lower amount of anthocyanidins and a higher amount of flavonols compared to *P. inflata* (Esfeld et al., 2018). Bee pollinators should be attracted to both species, as they are both bee pollinated, but the higher ratio of anthocyanidins:flavonols as well as other phenotypic parameters may lead to a preference of *P. inflata*. We expect hawkmoths to have no preference for either of these species, as they both do not exhibit a hawkmoth pollination syndrome in terms of color and scent. However, it is possible that the different morphologies of the two purple species (short tube vs. long tube) may affect hawkmoth preference. We set up behavioral assays to compare these two species with the three pollinator species. No significant difference in first choice was visible for any of the pollinator species (Fig. 3.8 A). However, comparing the total choices made by each pollinator for the 10 min duration of each experiment revealed significant differences between pollinators. Hawkmoths significantly preferred the long tubed *P. secreta* over *P. inflata* while the opposite was observed for bumblebees and solitary bees (Fig. 3.8 B). These differences between first choice and total choices could be explained by the different morphologies of the plant species. The first encounters the pollinators make with a plant are most likely based on differences in color or scent but when visiting the flowers for more foraging bouts, they prefer to visit flowers that have a morphology that makes it easier for them to access the nectar reward (short tubed *P. inflata* for bees and long tubed *P. secreta* for hawkmoths with a proboscis).

When analyzing encounters of pollinators with both colored species, we not only quantified first and total choices, but also the type of choices made (refer to Fig. 3.1 and Methods section "Behavioral experiments" for description of these types of choices). Hawkmoths significantly preferred *P. secreta* in all three classifications of choices (hovering, probing, foraging) while bumblebees showed significant differences only when foraging (Suppl. Tables S3.11 – S3.14). The same was observed for solitary bees, where they significantly preferred *P. inflata* only when foraging (Suppl. Tables S3.15 and S3.16). However, solitary bees foraged much more frequently (68/70 forages to *P. inflata*) than bumblebees (27/33 forages to *P. inflata*) in the same time period (Suppl. Tables S3.14 and S3.16). This demonstrates the differences in selection pressure that may arise from different pollinator species from the same pollinator guild. If solitary bees forage more extensively than bumblebees, pollen may be depleted in this system very quickly, possibly affecting outcrossing rates if pollinated by solitary bees.

Change in UV absorbance causes major shift in primary pollinator preference

As floral advertisement to pollinators is caused through a combination of differing traits in wild *Petunia* species, disentangling the effects of an individual trait on pollinator behavior can be done through mutation of a single gene. We compared *myb-fl* mutants to wildtype *Petunia* lines to demonstrate the effect an alteration in a single speciation gene has on pollinators. The hawkmoth *M. sexta*, the main pollinator of *P. axillaris* in the wild, was used in pairwise choice assays to determine whether changes in behavior can be elicited by a precise mutation in a single gene. First, the *myb-fl* mutants were compared to *P. axillaris* N wildtype plants. Second, the *myb-fl* mutants were compared to *P. secreta* plants as the phenotypic shift in UV and visible color displays an intermediate phenotype between the two natural species *P. axillaris* and *P. secreta*. As for the previous comparisons with wildtype *Petunia* species, behavioral measurements, such as first choice and different foraging behaviors were recorded.

All hawkmoth behavioral assays showed a significant difference in first choice (n=20) between the *myb-fl* mutant and *P. axillaris* N wildtype. They significantly preferred the wildtype flowers over the *myb-fl* mutant flowers (Fig. 3.9). The same was also observed for the total number of encounters with flowers documented as well as the individual encounter types (hovering, probing, foraging; see Suppl. Tables S3.11 and S3.12). *M. sexta*

hovered in front of, probed, and foraged for nectar more frequently in UV absorbent wildtype *P. axillaris* N than *myb-fl* mutants. The opposite trend was observed for the comparison of the *myb-fl* mutants with the *P. secreta* natural species; both flowers appear UV reflective, but *P. secreta* flowers have stronger visible purple pigmentation by anthocyanins. Hawkmoths did not distinguish between the *myb-fl* mutants and *P. secreta* for their first choice (Fig. 3.9), but in total choices (Fig. 3.10) and the other three encounter types preferred the *myb-fl* mutants (Suppl. Tables S3.11 and S3.12). This points towards UV color being a major visual parameter for decision making for hawkmoths, while visible color does not elicit the same intense response. Altering only UV color seems to be enough to completely shift the primary pollinator preference away from the mutant but does not suffice to make the mutant and *P. secreta* identical in the eyes of a hawkmoth pollinator.

Figure 3.9: Total number of first choices for pairwise behavioral assays with *myb-fl* mutants. Graphs show comparisons for all three pollinator species: *M. sexta*, *B. terrestris* and *O. cornuta* (n=20 pollinators per comparison, total n=300). Bars display the total number of first choices for each 10 min experiment (gray = *P. axillaris*, pink = *P. secreta*, light pink = *P. axillaris* N *myb-fl*, faint pink = *P. axillaris* P *myb-fl*). Plant species labels on the left side of the graphs show the different pairwise comparisons. Statistical differences between groups were calculated using an exact binomial test and are depicted by letters. Full statistics are represented in Suppl. Tables S3.12 (*M. sexta*), S3.14 (*B. terrestris*) and S3.16 (*O. cornuta*).

Given the subtle phenotypic pigmentation differences observed between the *myb-fl* mutants of the two *P. axillaris* lines (N and P; Fig. 3.2 B), behavioral assays were also

performed on the *P. axillaris* P mutants to account for any differences in pollinator behavior and plant lines. Most pairwise comparisons showed the same results as for the previous comparisons. Hawkmoths preferred the wildtype *P. axillaris* P plants over the *myb-fl* mutants for the total number of choices they made (Fig. 3.10) as well as for the individual types of encounters (hovering, probing, foraging; see Suppl. Tables S3.11 and S3.12). The preference in first choice showed the same tendency (Fig. 3.9). The comparison of *myb-fl* mutants to the *P. secreta* species also showed similar results for the *P. axillaris* N and P lines; hawkmoths exhibited preference for the *myb-fl* mutants for their first choice (Fig. 3.9) as well as for total choices (Fig. 3.10).

Subtle differences between *myb-fl* mutants evoke a response by hawkmoth pollinators

To determine whether the subtle color differences between the *P. axillaris* N and P *myb-fl* mutants were meaningful to hawkmoth preference, we performed pairwise choice assays using both mutants. Hawkmoths did not distinguish between the two mutant lines when making their first choices (Fig. 3.9), but did so for total choices (Fig. 3.10). A tendency towards the *P. axillaris* P *myb-fl* mutants can be detected for hovering and probing of the hawkmoths, but not for foraging bouts (Suppl. Tables S3.11 and S3.12). We previously demonstrated a difference in scent composition between the different *P. axillaris* lines (Fig. 3.4 F-H), which may also influence pollinator behavior in this comparison of the *myb-fl* mutants. Taken together, all comparisons with *P. axillaris* N and P *myb-fl* mutants demonstrate that hawkmoths can clearly distinguish between the UV absorbent *P. axillaris* wildtype flowers and UV reflective mutants. Distinguishing between the *myb-fl* mutant and the *P. secreta* natural species is also possible, though not in all cases. The same holds for choice assays between the two *myb-fl* mutants: making a distinction is possible for hawkmoths, but not for first choice. The *myb-fl* mutants may therefore represent an intermediate stage of flowers, that are no longer adapted to their original pollinator, but not yet completely adapted to a novel pollinator.

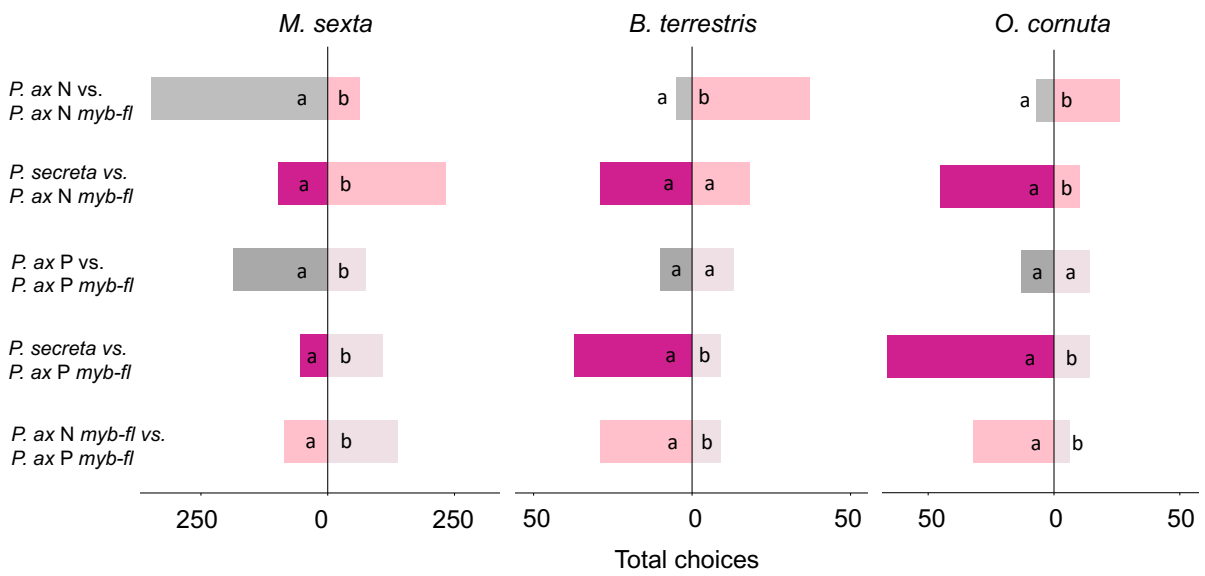

Figure 3.10: Total choices for pairwise comparisons with *myb-fl* mutants. Pairwise comparisons were run for all three pollinator species: *M. sexta*, *B. terrestris*, *O. cornuta* (n=20 pollinators per comparison, total n=300). Bars display the total amount of choices for each 10 min experiment (gray = *P. axillaris*, pink = *P. secreta*, light pink = *P. axillaris* N *myb-fl*, faint pink = *P. axillaris* P *myb-fl*). Plant species labels on the left side of the graphs show the different pairwise comparisons. Statistical differences between groups were calculated using an exact binomial test and are depicted by letters. Full statistics are represented in Suppl. Tables S3.12 (*M. sexta*), S3.14 (*B. terrestris*) and S3.16 (*O. cornuta*).

Phenotypic differences in UV color cause major shift in secondary pollinator behavior

If the *myb-fl* mutants represent an intermediate stage of adaptation towards a different pollinator group, it should be less attractive to the primary hawkmoth pollinator, and more attractive to a secondary or alternate pollinator. Bees are a possible secondary pollinator that have been observed visiting *P. axillaris* during the day (Hoballah et al., 2007). As natural *P. secreta* populations are pollinated by bees (Rodrigues et al., 2018b), we conducted the same pairwise comparisons with bumblebee and solitary bee pollinators, to demonstrate the preference of the secondary pollinator. Bumblebees showed a significant preference in first choice for the *P. axillaris* N *myb-fl* mutant over the *P. axillaris* N wildtype, opposite to what was observed for the hawkmoths (Fig. 3.9). Solitary bees showed no significant first choice preference for either species. When comparing the *myb-fl* mutant to the wildtype *P. secreta* line, bumblebees were still able to distinguish between the two and preferred *P. secreta* over the mutant for first choice. However, solitary bees did not exhibit preference between the two species when making

their first choice. These patterns were slightly altered when comparing the total choices of the two pollinator species, as bumblebees still significantly preferred the *myb-fl* mutant over *P. axillaris* N but did not prefer *P. secreta* over the *myb-fl* mutant (Fig. 3.10). Compared to the bumblebees, the solitary bees showed stronger preferences in their total choices. They preferred to visit the *myb-fl* mutant over *P. axillaris* N and *P. secreta* over the *myb-fl* mutant. These differences between the bee species, suggests that certain pollinators may adopt slightly different foraging strategies, with some being more selective during their first visits than others.

Differences in visible color of *myb-fl* mutant lines alter bee pollinator preference

Making use of the two different visible color phenotypes obtained with the different *myb-fl* mutant lines, we also tested the preference for the two bee pollinators with the faintly colored *P. axillaris* P *myb-fl* mutant. The pattern for the pairwise choice assays was slightly altered when comparing the this *myb-fl* mutant to the *P. axillaris* P and *P. secreta*. Here bumblebees as well as solitary bees did not significantly prefer the mutant over the wildtype for first choice, but did prefer *P. secreta* over the mutant when given the choice of these two plants (Fig. 3.9). The same pattern was visible when comparing the total choices of the assays (Fig. 3.10). These differences in choices observed when comparing the different *P. axillaris myb-fl* mutant lines may stem from the differences in visible coloration, where the *P. axillaris* N mutant is colored a darker shade of pink than the *P. axillaris* P mutant.

Given these differences observed for the two *myb-fl* mutant lines and that the *myb-fl* mutation induced a change in both the UV and visible pigmentation, making the detection of the individual effects of these two phenotypic changes difficult, we also decided to test the bees by presenting them with an array containing only the two mutants to choose from. *B. terrestris* and *O. cornuta* significantly preferred the darker colored *P. axillaris* N *myb-fl* mutant over the *P. axillaris* P *myb-fl* mutant for both first choice and total choices (Fig. 3.9 and 3.10). These differences are most likely due to visible color differences, but it must also be kept in mind that these flowers differ in their scent production, as not all *P. axillaris* species emit the exact same bouquet of scent volatiles (Fig. 3.4 F-H). As was found for all the hawkmoth experiments, the *myb-fl* mutant seems to represent an intermediate stage of the evolutionary process, where the primary (hawkmoth) and

secondary (bumblebee, solitary bee) pollinators prefer the *myb-fl* mutant over the wildtype, are able to distinguish between both *myb-fl* mutants, but the mutant and *P. secreta* wildtype is not yet indistinguishable.

UV light as causal factor for observed differences in pollinator preference

As we observed strong differences in hawkmoth preference between the wildtype *Petunia* species and the *P. axillaris* mutants, we set up experiments with different light regimes to assess whether the UV light was the causal factor for the observed differences. The same pairwise comparison as in previous experiments was set up (*P. axillaris* P vs *P. axillaris* P *myb-fl* mutant) but with pure visible light instead of including low amounts of UV light. Under these conditions hawkmoths were no longer able to distinguish between the two plant lines (Fig. 3.11, detailed statistics in Suppl. Table 3.17), demonstrating that differences in UV color of the flowers was indeed causing the differences in behavior of the pollinators. The second experiment set up was without any visible or UV light and using the two wild *Petunia* species *P. axillaris* N and *P. secreta*. We did this to control for the effect of artificial lighting being used to ensure that no altered behavior was provoked by this. Even though hawkmoths had no source of UV or visible light in the flight cage they were still able to distinguish between the two wild *Petunia* species, most likely due to other sensory cues such as scent (Fig. 3.11, detailed statistics in Suppl. Table 3.17). This underlines the importance of analyzing various floral cues that pollinators may use to distinguish between plant species and determining their individual contribution.

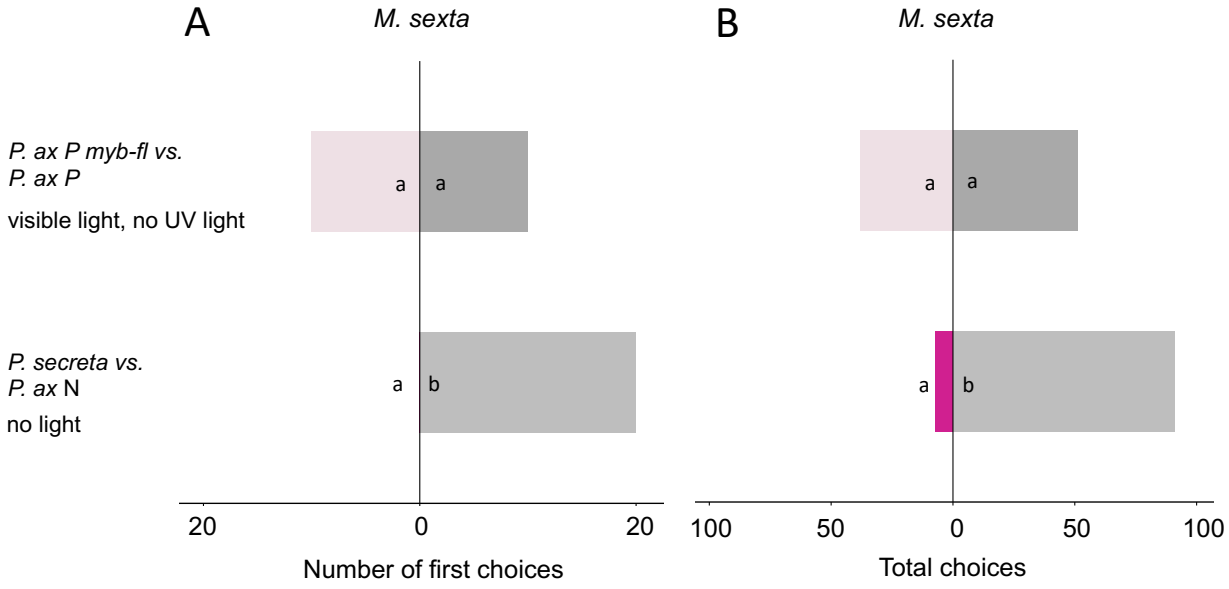

Figure 3.11: Light regime behavioral assays. (A) Percentage of first choice for pairwise comparisons of wildtype *Petunia* species with hawkmoth pollinators (*M. sexta*). Bars demonstrate the percentage of pollinator first choice (n=20 pollinators for each comparison). Hawkmoths could not distinguish between the wildtype and *myb-fl* mutant line if UV light was not provided during the experiment. They were still able to distinguish between the two wild plant species *P. axillaris* N and *P. secreta* if no light was provided at all during the experiment. Plant species labels on each side of the graphs show the different pairwise comparisons (pink = *P. secreta*, light pink = *P. axillaris P myb-fl,* gray = *P. axillaris*). (B) Total choices observed for the different light regime experiments. Moths did not exhibit preference in the experiment with visible light only when UV absorbance was the single factor different between the two species but were still able to detect differences between wild *Petunia* species if no light was provided. Statistical differences between groups were calculated using an exact binomial test for all data shown and are depicted by letters. Full statistics are represented in Suppl. Table S3.17.

Discussion

Shifts in pollinators have driven floral diversification and speciation in the angiosperms, and such scenarios often involve genes of large phenotypic effect. Increasing evidence of such genes in the literature provide insight into ongoing speciation processes (Hoballah et al., 2007; Chan et al., 2010; Amrad et al., 2016; Nadeau et al., 2016; Sheehan et al., 2016). However, testing the evolutionary role and specific effects of single gene mutations in speciation genes is difficult in many species. We achieved this by targeting the *MYB-FL* speciation gene using CRISPR/Cas9 through a precise mutation to demonstrate the impact this gene has on reproductive isolation through pollinator preference in *Petunia*.

MYB-FL mutation induces specific effects on genes of the flavonoid biosynthetic pathway

We observed downregulation of *MYB-FL* itself in the mutant lines after being inactivated through a mutation. This could possibly occur through the mechanism of nonsense mediated decay, a control mechanism that is conserved across eukaryotic cells to prevent translation of mutated mRNAs (Nickless et al., 2017). This could also explain why the non-functional *MYB-FL* is downregulated in the purple colored *P. secreta* (Esfeld et al., 2018). Despite the downregulation of *MYB-FL,* we observed few changes in expression of downstream genes involved in flavonol synthesis, with the exception of direct *MYB-FL* targets *FLS* and *HT1 (F3'H)*. Both *FLS* and *HT1 (F3'H)* seem to be directly activated through *MYB-FL* since the reduction in expression of *MYB-FL* led to a severe reduction in *FLS* and *HT1 (F3'H)* expression (Fig. 3.5). *MYB-FL* has been suggested to potentially have a repressor function for anthocyanin related genes (Sheehan et al., 2016) which could not be completely confirmed with our data as only few anthocyanin related genes were moderately upregulated due to the loss-of-function mutation in *MYB-FL* (Fig. 3.5). However, although *MYB-FL* was downregulated, there was still substantial residual gene expression, which could explain why anthocyanin genes were only moderately upregulated if being repressed by *MYB-FL*.

Contrasting patterns of flavonol and anthocyanidin absorbance

As UV and visible color pigments are produced through the same flavonoid biosynthetic pathway and we observed changes in gene expression of both flavonol and anthocyanin specific genes, we analyzed flavonol and anthocyanidin presence in our *myb-fl* mutants. Delphinidin and cyanidin were the two major anthocyanidin compounds detected in *myb-fl* mutant floral limb pigments (Fig. 3.3 A), despite the fact that the anthocyanin transcription factor controlling floral limb color in *Petunia*, *AN2*, is nonfunctional in *P. axillaris* (Esfeld et al., 2018). This naturally raises the question of the transcriptional mechanism that activates their production in the mutants. Since *AN2* has previously been demonstrated to be non-functional in *P. axillaris* lines (Esfeld et al., 2018), a different transcription factor may possibly be taking over the role of the non-functional *AN2* in the light or faint pink colored *myb-fl* mutants. A candidate would be *DPL*, which was co-opted to replace *AN2* in related *P. exserta* (Berardi et al., 2021). *DPL* was both expressed and unchanged in the wildtype *P. axillaris* plants and *myb-fl* mutants (Fig. 3.5). Alternatively

another transcriptional configuration of the complex may be activating anthocyanin production that has yet to be explored.

Not only did our phenotypic analyses show an increase in anthocyanidin absorbance but a simultaneous reduction in flavonol absorbance in the *myb-fl* mutants compared to wildtype plants (Fig. 3.3 B). The most likely explanation for the increase in visible color pigments in the *myb-fl* mutants is competition for shared substrates, the dihydroflavonols. We also observed differences in anthocyanidin absorbance between the *myb-fl* lines (Fig. 3.3 B). Although anthocyanidin absorbance values were very low in the *myb-fl* mutants the differences between the *P. axillaris* N and P *myb-fl* lines were strong enough to induce a phenotypic difference (Fig. 3.2 B). Through competition for shared substrates it would therefore follow that the line with more visibly colored pigments (*P. axillaris* N *myb-fl*) should display lower flavonol absorbance. We did not observe significant differences in flavonol absorbance between *P. axillaris* N and P *myb-fl* mutant lines, but the overall flavonol absorbance was higher than the anthocyanidin absorbance (Fig. 3.3 B). If subtle differences in anthocyanin production influence the visible color phenotype, equally subtle differences in flavonol production may occur through competition for shared substrates. However, due to the higher overall quantities of flavonols, these subtle differences may not be statistically significant. This direct mutation in the key transcription factor *MYB-FL* lets us further investigate the nature of substrate competition between anthocyanin and flavonol biosynthesis that has been proposed previously in transposon mutation lines (Sheehan et al., 2016).

Highly specific effects of transcription factor in metabolic network

Our observations of the few specific effects the *myb-fl* mutation had on gene expression in the flavonoid biosynthetic pathway led us to investigate how this mutation would alter gene expression on a global scale. We were able to detect 55 significantly differentially expressed genes in the *myb-fl* mutants (Suppl. Table 3.2) but no phenotypic effects on any traits potentially influencing pollinator behavior apart from floral color (Fig. 3.4) and therefore conclude that the function of *MYB-FL* is highly specific. Transcription factors involved in gene regulatory networks often have pleiotropic effects: promoters of key transcriptional regulators can be bound by multiple transcription factors and simultaneous activator as well as repressor functions have been found for these

transcription factors (Brady et al., 2011; Franco-Zorrilla et al., 2014; Taylor-Teeples et al., 2015; Gaudinier et al., 2018). The very specific activating effects of *MYB-FL* on *FLS* and *HT1 (F3'H)*, imply that this is not the case for *MYB-FL*, but that *MYB-FL* is a more specific transcription factor. How is this specificity on a genetic and phenotypic level achieved?

Genetic redundancy not likely for flavonol production

One possibility to achieve such specificity is genetic redundancy that helps buffer gene regulatory networks and makes them highly robust (Nawy et al., 2005; Brady et al., 2011). In this scenario, loss-of-function mutations in single transcription factors do not lead to any phenotypical changes when other functional transcription factors can take over. While a large phenotypic change was observed for *MYB-FL*, to our knowledge there is currently no genetic redundancy in *Petunia* that could take over flavonol production. The most likely candidates would be in the same subgroup 7 (SG7) of MYB transcription factors as *MYB-FL* and possibly under control of *MYB-FL*. There are only two other MYBs in this subgroup (Berardi et al., 2021) of which we were able to detect one (Peaxi162Scf00001g00231.1) in our RNAseq dataset (Suppl. Table S3.20). Expression of this MYB was extremely low (< 5 normalized read counts in both mutant and wildtype lines) and it did not show significant differential expression, ruling out the possibility of this being a suitable candidate. The second MYB was not expressed. Thus genetic redundancy of *MYB-FL* for flavonol production does not seem feasible in *Petunia*. However, genetic redundancy in other metabolic networks is still possible. No other phenotypic effects, e.g. defense against high light stress, were found as a result of the *myb-fl* mutation, which could point towards other aspects of the network *MYB-FL* is connected in, being buffered by other transcription factors. In fact we have proposed such a mechanism for *AN2*, where despite this transcription factor being non-functional in *P. axillaris*, anthocyanidin pigments were produced in the *myb-fl* mutants. A different transcription factor seems to be taking over the role of *AN2* in this scenario.

Precise expression patterns as a driver for transcription factor specificity

Another possibility for achieving specificity of transcription factors is precise temporal or spatial expression. If pleiotropic effects of transcription factors are possible when these factors are expressed, specific up- or downregulation of these factors can hinder such effects from occurring, resulting in transcription factor specificity in plant organs

(spatial) or developmental stages (temporal). We were able to detect spatial specificity of *MYB-FL* expression in floral organs in our RT-qPCR experiment (Fig. 3.7). *MYB-FL* was significantly downregulated only in the floral limb and pistils of the mutant lines. However, expression levels in the floral limb were two orders of magnitudes higher than in any other organ. This points towards spatially specific expression of *MYB-FL* in the floral limb during bud and flower development, which if downregulated results in a low amount of flavonols produced and leads to the observed UV-reflecting phenotype in mutants. This spatially specific expression pattern has also been found in other R2R3-MYB transcription factors regulating flavonol synthesis (Mehrtens et al., 2005; Stracke et al., 2007; Czemmel et al., 2009; Stracke et al., 2010; Zhong et al., 2020). In a previous study, expression of *MYB-FL* in *P. axillaris* increased by a factor of 5 across bud stages 1 - 5/6 during flower development (Sheehan et al., 2016), pointing towards an additional level of temporal control of expression of this transcription factor. It seems that a combination of spatial specificity coupled with temporally controlled expression determines specificity of *MYB-FL* in *Petunia* during floral development.

No negative effects on survival of the *MYB-FL* mutation with abiotic stressors

The production of flavonols in other species such as *Brassica*, *Helianthus* (sunflower) and *Arabidopsis* is also regulated by MYB transcription factors (Stracke et al., 2007; Brock et al., 2016; Todesco et al., 2021). Different UV pigmentation patterns have been found in these species that are not only associated with pollinator preference, but with environmental variables as well. Considering this dual role of UV pigmentation in pollinator attraction and abiotic responses, we analyzed possible negative fitness effects in the *myb-fl* mutants under high light stress. We focused on high light as an abiotic stressor for two reasons: (1) Genes potentially associated with light response were differentially expressed in our RNAseq dataset and (2) *P. axillaris* grows in sunny, high light environments (Turchetto et al., 2014, 2019). We did not observe any negative effects on chlorophyll content in the leaves (Fig. 3.6) nor on fitness-related factors, such as number of flowers produced or flowering time. Again the specificity of *MYB-FL* we observed most likely contributed to this finding. If the function of the *MYB-FL* gene is highly specific, then mutating it should not result in pleiotropic effects. Additionally the spatial specificity of *MYB-FL* expression we observed in floral organs (Fig. 3.7), hinders unwanted pleiotropic effects from occurring. *MYB-FL* expression levels were extremely

low in all organs except floral limbs and will most likely be unaffected by a loss-of-function mutation in *MYB-FL*. Further possible buffering effects in gene networks might help avoid unwanted responses to abiotic stressors if *MYB-FL* is mutated. As flavonoids play a key role for the survival and reproductive success of a plant, tightly regulating this pathway to avoid pleiotropic effects if mutations occur, seems to be an effective way to ensure plant survival.

Major shifts in pollinator preference due to a single speciation gene

Through pollinator choice assays, we have been able to demonstrate that a large contribution to reproductive isolation can occur through a mutation in a single major effect gene. Primary pollinators (hawkmoths) significantly preferred the wildtype plants over the *myb-fl* mutants (Fig. 3.9 and 3.10) demonstrating a strong reduction in primary pollinator preference of the mutant. However, this reproductive isolation was not yet perfect, as secondary pollinators (bees) were still able to distinguish between the *myb-fl* mutant and *P. secreta* flowers, although not in all cases (Fig. 3.9 and 3.10). Most likely, the *myb-fl* mutant represents an intermediate form of the evolutionary process that occurred during the shift from hawkmoth to bee pollination syndrome. A mutation of major phenotypic effect moves the plant away from its fitness optimum (hawkmoth pollination syndrome) towards a different fitness optimum (bee pollination syndrome), but it has not yet reached the peak of this second fitness optimum. In some scenarios such major phenotypic leaps may be deleterious, but this case demonstrates that a leap large enough to be almost fully adapted to a new pollinator, can allow speciation to occur through changes in a low number of genes of large effect. Even in nature, visitation by pollinators to *Petunia* species is not exclusive to a single pollinator. For example *P. axillaris* is mainly visited by nocturnal hawkmoths, but also receives bee visits during the day (Hoballah et al., 2007). For reproductive isolation to occur, a strong but not perfect pollinator preference may therefore suffice.

Specific pollinator composition affects pollination success

How this large mutation will affect reproductive isolation also strongly depends on the pollinator assemblage present in the environment. We observed differences in behavior between the two bee species (bumblebees *B. terrestris* and solitary bees *O. cornuta*) we used, demonstrating possible positive or negative fitness effects on plant reproduction

depending on the pollinator species. Solitary bees were more selective when foraging, which may have possible implications for plant reproduction. It was during foraging bouts that solitary bees were observed loading their pollen sacs with large amounts of pollen while also foraging for nectar on *P. inflata*, which may lead to a higher pollen load being depleted from the plant after a single foraging bout. Wild *P. secreta* populations are pollinated by halictid bees of the genus *Pseudogapostemon* (Rodrigues et al., 2018b). Bees of this genus have been observed to deplete 86% of the pollen after only a single foraging bout to *Petunia mantiqueirensis* (De Araujo et al., 2020). These pollinators also almost exclusively visited plants the first day after anthesis (De Araujo et al., 2020), demonstrating that their behavior is adapted to pollen foraging and that this may exert very specific pressures on plants when to have stigmas receptive to pollen if visits occur only in a very narrow time window.

If the *Pseudogapostemon* bees in the wild deplete high amounts of pollen after their first visit to plants, there are two possible scenarios for the plant that could result from this. One possibility would be that the pollinators return to their nests with the pollen loads after visiting only a single or few flowers, deposit their pollen load and return for more foraging afterwards, essentially depleting the pollen from the plants without providing any pollination service. In this scenario if 86% of pollen is depleted after a single round of foraging, this would negatively affect outcrossing rates. The other possibility would be that bees visit many other flowers in succession with large pollen loads in their scopa, allowing them to deposit pollen extensively on flowers and positively affecting outcrossing rates. This is the more likely scenario as *Pseudogapostemon* bees were often observed visiting flowers carrying pollen in their scopa (De Araujo et al., 2020). These differences in foraging strategies and pollinator behavior, not just during, but also in between and after flower visits, underline the importance of considering differences even in pollinators of the same guild.

UV color as an initial cue for hawkmoth preference

Comparing the *myb-fl* mutants not only to *P. axillaris* wildtype plants but also to *P. secreta* wildtype plants allowed us to study the effect of UV color on pollinator preference in more detail. Hawkmoths did not exhibit preference between *P. secreta* and *myb-fl* mutants for first choice but did so for total choices where they preferred the *myb-fl* mutants (Fig. 3.9

and 3.10). Based on these findings UV color seems to act as an initial cue during a hawkmoth's decision making process to draw a pollinator to a flower, as these two plant lines did not differ in UV color (both reflective) and the hawkmoths were therefore not able to use this cue to distinguish between the two. This is also supported by all the experimental setups where the plant lines solely differed in UV color (absorbent vs. reflective) and hawkmoths strongly preferred UV absorbent flowers. Given these results it follows that no preference was found between reflective species *P. secreta* and *P. inflata* for first choice while significant preferences were found for total choices (Fig. 3.8). Again this points towards UV color being an initial cue where hawkmoths are not able to distinguish between the flowers for first choice if they display the same UV color phenotype, but can display preferences for total choices due to other cues. One could argue that this observation results from limited statistical power of first choice experiments as a maximum of one first choice can be made by each pollinator, while they can encounter many flowers for their total choices during 10 min trials. This could be considered a caveat if the same trend observed for first choice experiments for each pollinator species was subsequently observed for total choices but more pronounced, leading to statistical significance. However, this was not the case for all pollinator species: bumblebees showed a near 1:1 visitation rate for first choices when presented with *P. inflata* and *P. secreta* plants, while this trend clearly shifted in favor of *P. inflata* for total choices. Ideally to reduce this possible caveat of statistical power an even larger number of first choice experiments with pollinators would need to be set up.

Multiple cues needed for a complete shift in pollinator preference

Pollinators can integrate multiple cues to choose between plant species. Just like color, scent differences between species also affect pollinator preference. This should not have influenced our comparisons of the *myb-fl* mutants and *P. axillaris* lines, as these only differed in color. However, this may have played a role in the comparisons between the *myb-fl* mutants and *P. secreta*. Previous analyses of hawkmoth preferences for *Petunia* scent volatiles discovered that hawkmoths preferred scented to non-scented plants, but when presented with conflicting cues (scent and color), both cues were equivalent (Klahre et al., 2011). This demonstrates that a combination of cues may be necessary to make efficient choices. In our setup *P. secreta* produces a very low amount of scent volatiles (A. Berardi, unpublished data) while the *myb-fl* mutants still produce scent,

which may affect the hawkmoth's choice to preferentially visit the mutants. Additionally altering the scent profile in the *myb-fl* mutant plant could help achieve a complete shift towards the secondary pollinator.

Once pollinators have approached a flower there are additional cues that affect a pollinator's behavior. Bumblebees are able to use touch-based discrimination to distinguish between different surfaces (Whitney et al., 2009). This has not been found for hawkmoths but based on the way hawkmoths and bees forage (hovering vs. landing on a flower), tactile cues are likely to be less important for hawkmoths. Conical epidermal cells between wild *Petunia* lines were not all shaped the same (Fig. 3.4 I), which may be optimized to allow bees to access the floral reward in the bee pollinated species. Bees have been observed to discriminate against mutants lacking conical cells and have an easier time physically handling plants with conical cells for flower surfaces that are difficult to manipulate (Comba et al., 2000; Whitney et al., 2009). Multiple layers of cues may therefore influence a pollinator's decision making process and could explain differences between pollinator guilds.

Reproductive isolation through multiple major effect genes

The *Petunia* system is ideal to evaluate the effect of a single mutated gene of major phenotypic effect at the level of reproductive isolation through both primary and secondary pollinator preference. Mutating *myb-fl* resulted in UV reflective flowers while simultaneously freeing up precursors in the pathway to produce anthocyanins. This trade-off between flavonols and anthocyanins puts a restriction on the order of color changes in *Petunia*. In order for purple pigmented *P. secreta* to evolve from a colorless ancestor, a decrease in flavonols is required before anthocyanins can be produced. Therefore the *myb-fl* mutation most likely occurred at an earlier time point during the shifts from hawkmoth to bee pollination syndrome than changes in visible color. This dual phenotypic effect on UV and visible color allows *P. axillaris* to move away from its fitness optimum and deter the primary pollinator while simultaneously allowing the attraction of a new pollinator guild. This is supported by our experiments showing that bumblebees and solitary bees are able to perceive even subtle differences in visible color phenotypes between the two *myb-fl* mutant lines and significantly prefer the darker colored lines (Fig. 3.9 and 3.10). For bee pollinators the detectability of a flower can be

increased or decreased by UV reflectance depending on the reflectance that occurs in other parts of a bee's visibility spectrum (Kevan et al., 2001) and for white flowers UV reflectance can significantly prolong a bee's search time (Spaethe et al., 2001). Only altering UV color through the *MYB-FL* mutation would most likely not be sufficient to effectively attract a new pollinator guild, such as bees and maintain the mutation in a population. The loss-of-function mutation in *MYB-FL* therefore simultaneously represents a gain-of-function mutation through the flavonol/anthocyanin trade-off in the flavonoid pathway. Through its dual phenotypic effect this single gene is enough to strongly contribute towards reproductive isolation of the *P. axillaris* and *P. secreta*. The effects of this single gene can now be extrapolated to study the effects of multiple major effect genes on reproductive isolation in *Petunia*.

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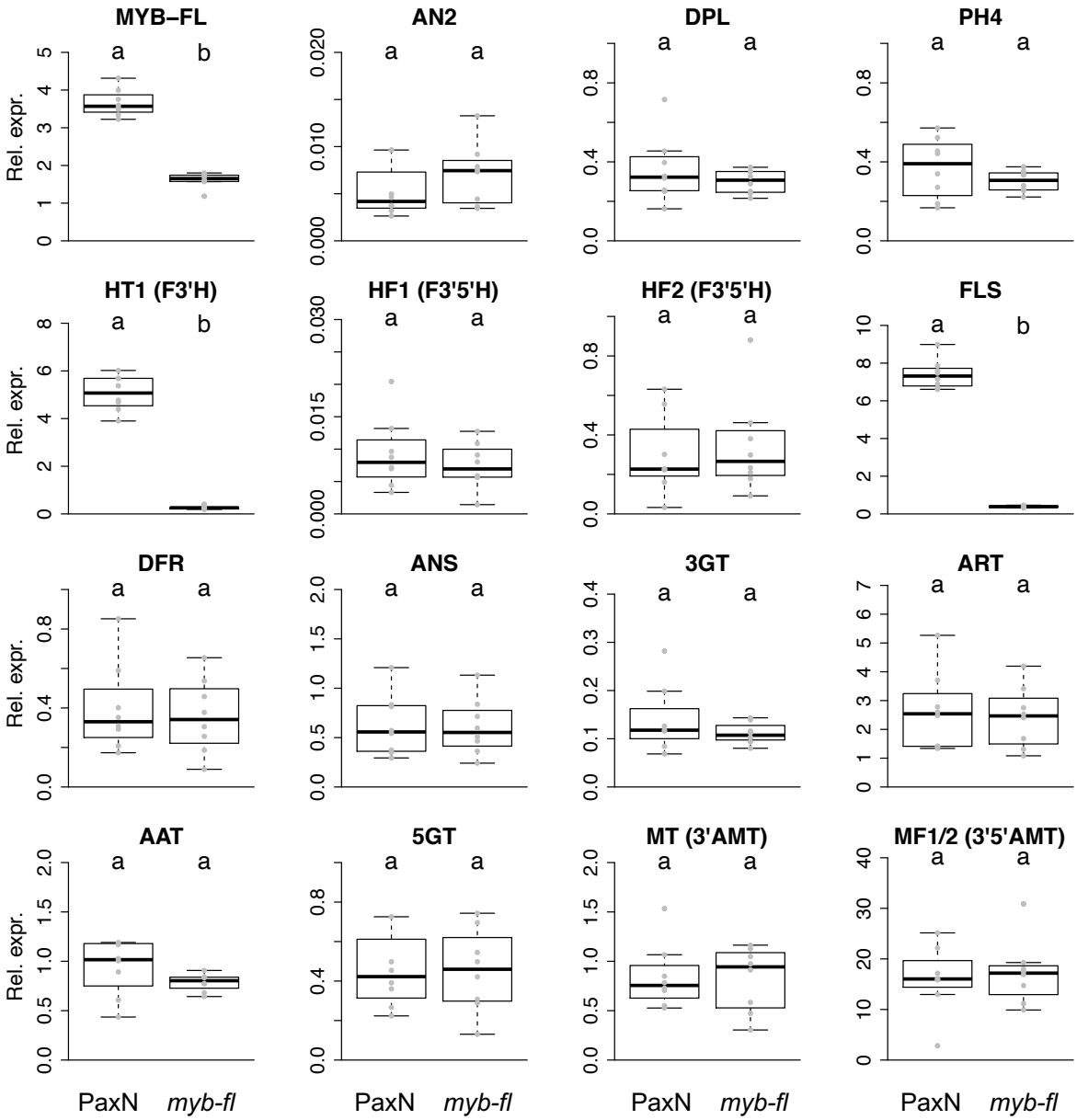
Supplemental data



Supplemental Figure S3.1: RT-qPCR of genes influencing flavonoid synthesis in the *myb-fl* mutant and wildtype *P. axillaris* N. Expression of genes in the flavonoid biosynthetic pathway in *P. axillaris* N wildtype lines compared to *myb-fl* mutants as obtained by RT-qPCR (stage 4 petal limb tissue). Boxplots represent medians of eight biological replicates. Values are represented as the mean relative expression of the target gene relative to three reference genes *SAND*, *RAN1* and *ACTIN11* (Mallona et al., 2010). Pairwise comparisons of groups were tested for their significance with a Mann-Whitney U test. Differences in significances are represented by letters.

Supplemental Table S3.1. Log2 fold change (L2FC) values and statistics for RNAseq of flavonoid biosynthetic pathway genes. Expression in petal limbs (bud developmental stage 4) for the RNAseq dataset from *P. axillaris* and *P. axillaris myb-fl* mutants, filtered for genes in the flavonoid biosynthetic pathway.

Gene info			**Log2 fold change (L2FC)**		**Normalized read counts**	
Gene ID	Chromosome (*P. ax* v402)	*Petunia* gene name	L2FC[a]	padj L2FC	Average *axillaris*	Average *myb-fl*
Peaxi162Scf00858g00215	Chr2	PAL-A	0.176988077	0.67349143	6311.711214	5583.094471
Peaxi162Scf00488g00074	Chr7	PAL-B	0.124395827	0.782072767	2047.826228	1878.051104
Peaxi162Scf00123g00096	Chr4	PAL-C	1.818219735	5.18E-116	26688.9383	7567.987561
Peaxi162Scf00556g00035	Chr6	C4H-A	-0.038123563	0.895360375	2700.250504	2771.393794
Peaxi162Scf00390g00225	Chr3	C4H-B	-0.031544983	0.947886668	9095.111997	9295.789428
Peaxi162Scf00314g00086	Chr3	4CL-A	0.053108667	0.926836968	789.6815995	760.9672005
Peaxi162Scf00195g01223	Chr1	4CL-B	0.056699548	0.972352636	7.045672991	6.522175003
Peaxi162Scf00610g00346	Chr2	4CL-C	-0.052636813	0.953355939	35.72985241	37.14362063
Peaxi162Scf00207g00334	Chr1	4CL-D	0.189440941	0.715785082	1334.156533	1170.069266
Peaxi162Scf00047g01225	Scf02009	CHS-A	1.954204816	1.27E-203	70425.33225	18174.32165
Peaxi162Scf00536g00092	Chr2	CHS-J	2.15801712	0.0000000000000292	6599.776302	1478.873947
Peaxi162Scf00006g00088	Chr5	CHI-A	1.712250078	3.11E-61	14690.63881	4483.158391
Peaxi162Scf00328g01214	Chr4	F3H	1.146924351	6.89E-23	29142.64158	13160.48841
Peaxi162Scf00201g00243	Chr3	HT1 (F3'H)	3.986878605	1.06E-214	26333.01193	1661.03128
Peaxi162Scf00150g00218	Chr1	HF1 (F3'5'H)	-0.220231467	0.846933045	75.36424863	88.06894299
Peaxi162Scf00108g00417	Chr5	HF2_1 (F3'5'H)	-1.561983974	0.000214108	257.7678379	760.8186381

Peaxi162Scf00081g01223	Chr4	DIF-F	-1.004123935	0.008082917	1749.858025	3510.590126
Peaxi162Scf00927g00035	Chr2	FLS	3.669603588	6.54E-115	38318.08551	3011.347362
Peaxi162Scf00366g00630	Scf04944	DFR	-0.905636131	0.056514134	426.7293258	799.8126495
Peaxi162Scf00620g00533	Chr7	ANS	-0.887856615	0.001907256	3837.781022	7101.778529
Peaxi162Scf00163g00081	Chr7	3GT	0.237570758	0.673095446	1396.310441	1184.476037
Peaxi162Scf00487g00064	Chr6	RT	-0.482175268	0.360675399	6338.22432	8854.005018
Peaxi162Scf00160g00833	Chr5	AAT	0.479905641	0.450653873	2788.919293	1999.960706
Peaxi162Scf00378g00113	Chr5	5GT	-0.447751988	0.561939608	1706.964974	2328.575826
Peaxi162Scf00518g00430	Chr5	MT (3'AMT)	-0.340677645	0.63560878	379.7417238	481.5925619
Peaxi162Scf00089g00427	Chr3	MF1 (3'5'AMT)	-1.130675204	0.017120573	587.2829235	1285.648391
Peaxi162Scf00316g00055	Chr5	MF2 (3'5'AMT)	-0.820594025	0.0080331	19864.64719	35083.2033
Peaxi162Scf00450g00031	Chr4	CcoAOMT2	0.121028821	0.861396286	777.4328972	714.9992989
Peaxi162Scf00450g00032	Chr4	CcoAOMT3	0.050353358	0.940426215	169.2318853	163.7386206
Peaxi162Scf00119g00942	Chr7	JAF13	-0.149520181	0.523796642	1292.374938	1433.366702
Peaxi162Scf00016g02023	Chr6	CcoAOMT1	0.111264657	0.772911957	2489.817287	2305.008487
Peaxi162Scf00118g00310	Chr6	AN2	-0.849205185	0.366558854	63.86670084	115.192821
Peaxi162Scf00886g00028	Chr2	MYB-FL	0.721091818	0.0000177	7231.777468	4386.513825
Peaxi162Scf00338g00912	Chr6	AN1	-0.155734198	0.550634891	3451.497195	3843.658734
Peaxi162Scf00912g00146	Chr3	AN11	-0.383323129	0.146581666	832.4414428	1084.779197
Peaxi162Scf00713g00038	Chr1	AN9	0.0444077	0.961260351	2538.947156	2462.245004
Peaxi162Scf01210g00002	Chr7	DPL	-0.302390287	0.552050168	429.6439112	530.3824241

Peaxi162Scf03779g00019	Scf07696	MYB27	0.013589129	0.9761607	668.3586217	661.9867173
Peaxi162Scf00521g00814	Chr6	MYBX	-0.701596381	0.598623972	123.9069954	201.7478827
Peaxi162Scf00569g00024	Chr1	PH1	-1.018107022	0.008874788	219.9696256	444.9892567
Peaxi162Scf00472g00077	Scf04858	PH3	-0.648563291	0.021904581	1990.559708	3120.247274
Peaxi162Scf00349g00057	Chr3	PH4	-0.137192971	0.499181216	458.4133614	502.7270654
Peaxi162Scf00177g00620	Chr1	PH5	-1.587060944	0.000000333	1038.631484	3120.549866
Peaxi162Scf00658g00110	Scf04850	PHZ	-0.262349816	0.76388598	583.0540024	699.0145524
Peaxi162Scf00071g00030	Chr2	Fading	0.534822651	0.620166981	186.9215822	128.9874581
Peaxi162Scf00003g02440	Chr7	CcoAOMT4	-0.000994709	0.998055391	586.295002	586.7330077
Peaxi162Scf00003g02446	Chr7	CcoAOMT5	0.578339168	0.518158799	19.49488131	12.79368963

a) Log2 fold-change was calculated between *P. axillaris* and *P. axillaris myb-fl* mutants stage 4 petal limbs; positive values indicate *P. axillaris* > *P. axillaris myb-fl* and negative values indicate *P. axillaris* < *P. axillaris myb-fl*

Supplemental Table S3.2. Filtering for differentially expressed genes in RNAseq dataset. Differential expression in petal limbs (bud developmental stage 4) for the RNAseq dataset from *P. axillaris* and *P. axillaris myb-fl* mutants, filtered for genes with a L2FC of ±1 and adjusted p-value < 0.05.

Gene info			**Log2 fold change**		**Normalized read counts**		**Functional annotations**			
Gene ID	Chromosome (*P. ax* v402)	*Petunia* gene name	L2FC[a]	padj L2FC	Average *axillaris*	Average *myb-fl*	BLASTP	PFAM	INTERPRO	GO
Peaxi162Scf00107g01115	Chr6		4.051	0.0127	334.792	20.240	sp|Q8GXU5|SDI1_ARATH	Tetratricopeptide repeat	Tetratricopeptide repeat	GO:0005515 protein amino acid binding, glycoprotein binding
Peaxi162Scf00201g00243	Chr3	HT1	3.987	1.06E-214	26333.012	1661.031	sp|Q9SBQ9|F3PH_PETHY	Cytochrome P450	Cytochrome P450	GO:0005506|GO:0016705|GO:0020037|GO:0055114 iron ion binding, oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, oxidation-reduction process
Peaxi162Scf00045g00142	Chr2		3.837	6.53E-32	308.935	21.574	sp|Q0JKZ0|SNL6_ORYSJ	3-beta hydroxysteroid dehydrogenase/ isomerase family	3-beta hydroxysteroid dehydrogenase/ isomerase	GO:0003854|GO:0006694|GO:0016616|GO:0055114 3-beta-hydroxy-delta5-steroid dehydrogenase activity, steroid biosynthetic process, oxidoreductase activity
Peaxi162Scf00927g00035	Chr2	FLS	3.670	6.54E-115	38318.086	3011.347	sp|Q07512|FLS_PETHY	non-haem dioxygenase in morphine synthesis N-terminal	Non-haem dioxygenase N-terminal domain	
Peaxi162Scf00167g01221	Chr2		3.045	4.86E-57	13258.164	1605.531	sp|Q8VZW3|CFI3_ARATH	Chalcone-flavanone isomerase	Chalcone isomerase	GO:0016872 intramolecular lyase activity
Peaxi162Scf01650g00008	Chr3		2.923	0.0223	394.699	52.094	sp|Q9FIR9|LSU2_ARATH			
Peaxi162Scf00848g00224	Chr1		2.915	7.31E-291	6620.085	877.210	sp|F4JTB3|DTX35_ARATH	MatE	Multi antimicrobial extrusion protein	GO:0006855|GO:0015238|GO:0015297|GO:0016020|GO:0055085

										drug transmembrane transport, antiporter activity, membrane transport, ATP hydrolysis coupled transmembrane transport
Peaxi162Scf00248g00003	Chr2		2.892	0	4468.400	601.607	sp|Q53UH4|DUSKY_IPONI	UDP-glucoronosyl and UDP-glucosyl transferase	UDP-glucuronosyl/UDP-glucosyltransferase	GO:0016758 hexosyltransferase activity
Peaxi162Scf00540g00013	Chr3		2.534	0.0137	258.521	44.592	sp|O24661|ASNS_TRIVS	Asparagine synthase	Asparagine synthase	GO:0004066|GO:0006529 asparagine synthase (glutamine-hydrolyzing) activity, asparagine biosynthetic process
Peaxi162Scf00016g01738	Chr6		2.336	5.63E-102	3794.235	751.031	sp|Q5NUF4|HIDM_GLYEC	alpha/beta hydrolase fold	Alpha/beta hydrolase fold-3	GO:0016787 hydrolase activity
Peaxi162Scf00366g00216	Chr4		2.325	4.35E-21	292.897	58.234	sp|A8GCT3|RUTD_SERP5	alpha/beta hydrolase fold	Alpha/beta hydrolase fold-1	
Peaxi162Scf00536g00092	Chr2	CHS-J	2.158	2.92E-14	6599.776	1478.874	sp|P22928|CHSJ_PETHY	Chalcone and stilbene synthases, N-terminal domain	Chalcone/stilbene synthase, N-terminal	
Peaxi162Scf00332g00629	Chr3		2.031	6.53E-32	174.935	43.000	sp|Q949X0|ADS3_ARATH	Fatty acid desaturase	Fatty acid desaturase domain	GO:0006629 lipid metabolic process
Peaxi162Scf00047g01225	Scf02009	CHS-A	1.954	1.27E-203	70425.332	18174.322	sp|P08894|CHSA_PETHY	Chalcone and stilbene synthases, N-terminal domain	Chalcone/stilbene synthase, N-terminal	
Peaxi162Scf00123g00096	Chr4	PAL-C	1.818	5.18E-116	26688.938	7567.988	sp|P25872|PAL1_TOBAC	Aromatic amino acid lyase	Aromatic amino acid lyase	
Peaxi162Scf00323g00052	Chr5		1.810	2.35E-14	189.985	54.211	sp|Q9SJ02|STEP2_ARATH			
Peaxi162Scf01340g00216	Chr7		1.732	3.41E-40	4149.005	1249.548	sp|Q9SX83|DTX33_ARATH	MatE	Multi antimicrobial extrusion protein	GO:0006855|GO:0015238|GO:0015297|GO:0016020|GO:0055085 drug transmembrane transport, antiporter activity, membrane transport, ATP hydrolysis coupled transmembrane transport

Peaxi162Scf00006g00088	Chr5	CHI-A	1.712	3.11E-61	14690.639	4483.158	sp|Q9LTT3|HMG10_ARATH	ARID/BRIGHT DNA binding domain	ARID DNA-binding domain	GO:0003677 DNA binding
Peaxi162Scf00074g01119	Chr7		1.673	0.0428	286.489	89.806				
Peaxi162Scf00003g00251	Chr7		1.624	6.09E-27	439.019	142.573	sp|Q2V6K1|UGT_FRAAN	UDP-glucoronosyl and UDP-glucosyl transferase	UDP-glucuronosyl/UDP-glucosyltransferase	GO:0016758 hexosyltransferase activity
Peaxi162Scf00284g00234	Chr3		1.522	0.0002	600.029	208.580	sp|Q94AR4|CID2_ARATH	Ataxin-2 C-terminal region	Ataxin-2, C-terminal	
Peaxi162Scf00047g01222	Chr5		1.488	9.62E-09	1484.141	529.180	sp|Q9FKB0|LSM5_ARATH	LSM domain	LSM domain, eukaryotic/archaea-type	
Peaxi162Scf00314g01029	Scf04371		1.486	1.41E-05	642.085	228.961				
Peaxi162Scf00034g01311	Chr1		1.458	1.53E-24	916.706	333.963	sp|O48652|UVR3_ARATH	DNA photolyase	DNA photolyase, N-terminal	
Peaxi162Scf00213g01024	Chr7		1.379	1.04E-37	1868.539	718.288	sp|Q38JU2|CRYD_SOLLC	FAD binding domain of DNA photolyase	Cryptochrome/DNA photolyase, FAD-binding domain	
Peaxi162Scf01012g00124	Chr6		1.342	7.66E-28	469.507	185.223	sp|Q944H5|MTM1_ARATH	Mitochondrial carrier protein	Mitochondrial substrate/solute carrier	
Peaxi162Scf00160g00529	Scf02723		1.330	1.20E-11	251.317	100.197	sp|Q9C7D6|SCP17_ARATH	Serine carboxypeptidase	Peptidase S10, serine carboxypeptidase	GO:0004185|GO:0006508 serine-type carboxypeptidase activity, proteolysis
Peaxi162Scf00160g01527	Chr5		1.305	3.54E-17	3772.998	1526.920	sp|Q38970|ACC1_ARATH	Biotin carboxylase C-terminal domain	Biotin carboxylase, C-terminal	
Peaxi162Scf00493g00432	Chr3		1.290	1.17E-29	709.872	290.433	sp|Q9M682|GMK2_ARATH	Guanylate kinase	Guanylate kinase/L-type calcium channel beta subunit	
Peaxi162Scf00516g00671	Chr3		1.250	4.21E-06	830.524	349.256	sp|Q0JKZ0|SNL6_ORYSJ			
Peaxi162Scf01056g00030	Chr4		1.180	0.0175	171.608	75.445				
Peaxi162Scf00723g00019	Chr1		1.177	4.68E-12	716.535	317.092	sp|P85524|KIRO_ACTDE	Pathogenesis-related protein Bet v I family	Bet v I/Major latex protein	GO:0006952 defence response, physiological defense response, antimicrobial peptide activity,

										defense/immunity protein activity
Peaxi162Scf00078g00103	Chr2		1.172	1.53E-24	277.115	123.645		Uncharacterized protein conserved in bacteria (DUF2062)	Domain of unknown function DUF2062	
Peaxi162Scf00328g01214	Chr4	F3H	1.147	6.89E-23	29142.642	13160.488	sp|Q07353|FL3H_PETHY	non-haem dioxygenase in morphine synthesis N-terminal	Non-haem dioxygenase N-terminal domain	
Peaxi162Scf00095g00120	Chr5		1.126	0.0048	434.154	198.847	sp|Q8LBI9|EDL16_ARATH	Sugar (and other) transporter	Major facilitator, sugar transporter-like	GO:0016021|GO:0022857|GO:0055085 integral component of membrane, transmembrane transporter activity
Peaxi162Scf00271g00133	Chr7		1.114	3.26E-56	1236.664	571.861	sp|Q9SB00|PHR_ARATH			
Peaxi162Scf00700g00139	Chr6		1.097	1.05E-12	354.719	165.795	sp|Q84P23|4CLL9_ARATH	AMP-binding enzyme C-terminal domain	AMP-binding enzyme, C-terminal domain	
Peaxi162Scf00362g00431	Chr6		1.079	0.0072	158.344	74.807	sp|F4JTP5|STY46_ARATH	ACT domain	ACT domain	
Peaxi162Scf00038g00721	Chr2		1.069	0.0042	1890.934	901.228	sp|Q84N34|HHP2_ARATH	Haemolysin-III related	AdipoR/Haemolysin-III-related	GO:0016021 integral component of membrane
Peaxi162Scf00117g00321	Chr5		1.065	0.0425	139.176	66.552	sp|O82485|OPT7_ARATH	OPT oligopeptide transporter protein	Oligopeptide transporter, OPT superfamily	GO:0055085 transmembrane transport
Peaxi162Scf00071g00742	Chr2		1.062	9.91E-08	230.281	110.181	sp|Q3ECP7|ERDL5_ARATH	Sugar (and other) transporter	Major facilitator, sugar transporter-like	GO:0016021|GO:0022857|GO:0055085 integral component of membrane, transmembrane transporter activity
Peaxi162Scf00527g00733	Chr4		1.032	0.0488	244.502	119.640	sp|O81208|OHP1_ARATH			
Peaxi162Scf00166g01045	Chr4		1.022	3.29E-12	1161.827	572.847	sp|Q6R8G7|PHO13_ARATH	EXS family	EXS, C-terminal	GO:0016021 integral component of membrane

Peaxi162Scf00081g01223	Chr4	DIF-F	-1.004	0.0081	1749.858	3510.590	sp|O04354|CYB5_BOROF	Cytochrome b5-like Heme/Steroid binding domain	Cytochrome b5-like heme/steroid binding domain	
Peaxi162Scf00569g00024	Chr1	PH1	-1.018	0.0088	219.970	444.989	sp|P0ABB8|ATMA_ECOLI	haloacid dehalogenase-like hydrolase		
Peaxi162Scf00328g00118	Chr4		-1.033	0.0171	1806.916	3696.066				
Peaxi162Scf00089g00427	Chr3	MF1	-1.131	0.0171	587.283	1285.648				
Peaxi162Scf00047g02333	Chr5		-1.190	2.09E-06	526.905	1201.928	sp|Q9LYT3|DTX41_ARATH	MatE	Multi antimicrobial extrusion protein	GO:0006855|GO:0015238|GO:0015297|GO:0016020|GO:0055085 drug transmembrane transport, antiporter activity, membrane transport, ATP hydrolysis coupled transmembrane transport
Peaxi162Scf00175g00330	Chr1		-1.276	0.0091	69.739	169.047	sp|Q9FME2|NTF2_ARATH	Nuclear transport factor 2 (NTF2) domain	Nuclear transport factor 2	
Peaxi162Scf00560g00124	Chr2		-1.515	0.0001	258.701	739.547	sp|P31583|RHN1_NICPL	Ras family	Small GTPase	GO:0003924|GO:0005525 GTPase activity, GTP binding
Peaxi162Scf00108g00417	Chr5	HF2	-1.562	0.0002	257.768	760.819	sp|P48419|C75A3_PETHY	Cytochrome P450	Cytochrome P450	GO:0005506|GO:0016705|GO:0020037|GO:0055114 iron ion binding, oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, oxidation-reduction process
Peaxi162Scf00177g00620	Chr1	PH5	-1.587	3.33E-07	1038.631	3120.550	sp|Q43128|PMA10_ARATH	E1-E2 ATPase		
Peaxi162Scf00007g00636	Chr2		-1.618	0.0004	51.775	158.782	sp|P09789|GRP1_PETHY			
Peaxi162Scf00424g00414	Chr2		-1.993	3.58E-84	225.776	895.269	sp|Q9M8S6|SKOR_ARATH	Ion transport protein	Ion transport domain	GO:0005216|GO:0006811|GO:0016020|GO:0055085

										ion channel activity, ion transport, membrane, transmembrane transport
Peaxi162Scf00047g90144	Chr5		-2.323	3.99E-10	49.301	246.649				

a) Log2 fold-change was calculated between *P. axillaris* and *P. axillaris myb-fl* mutants stage 4 petal limbs; positive values indicate *P. axillaris* > *P. axillaris myb-fl* and negative values indicate *P. axillaris* < *P. axillaris myb-fl*

Supplemental Table S3.3. Statistics of phenotypic measurements of organ lengths and corolla area in *myb-fl* mutant lines (flowers one day post anthesis). Statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons. Groupings of the *myb-fl* mutants with their respective wildtype are shown in bold font.

Organ	**Comparison**	**p-value** (adjusted with Tukey *post hoc* comparisons)
Pistil length	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.0056763
	P. ax N myb-fl* vs. *P. ax N	**0.7326938**
	P. ax N myb-fl vs. *P. ax P*	0.0137518
	P. ax P myb-fl vs. *P. ax N*	0.0077482
	P. ax P myb-fl* vs. *P. ax P	**0.8339719**
	P. ax N vs. *P. ax P*	0.0192885
Stamen 1 length	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.0033346
	P. ax N myb-fl* vs. *P. ax N	**0.7676843**
	P. ax N myb-fl vs. *P. ax P*	0.0081934
	P. ax P myb-fl vs. *P. ax N*	0.0042370
	P. ax P myb-fl* vs. *P. ax P	**0.7491420**
	P. ax N vs. *P. ax P*	0.0106690
Stamen 2 length	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.0035609
	P. ax N myb-fl* vs. *P. ax N	**0.8909789**
	P. ax N myb-fl vs. *P. ax P*	0.0087727
	P. ax P myb-fl vs. *P. ax N*	0.0042292
	P. ax P myb-fl* vs. *P. ax P	**0.7551667**
	P. ax N vs. *P. ax P*	0.0106010
Stamen 3 length	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.0040523
	P. ax N myb-fl* vs. *P. ax N	**0.7929452**
	P. ax N myb-fl vs. *P. ax P*	0.0102674
	P. ax P myb-fl vs. *P. ax N*	0.0051623
	P. ax P myb-fl* vs. *P. ax P	**0.7339600**
	P. ax N vs. *P. ax P*	0.0134265
Stamen 4 length	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.0026917
	P. ax N myb-fl* vs. *P. ax N	**0.6460213**
	P. ax N myb-fl vs. *P. ax P*	0.0069530
	P. ax P myb-fl vs. *P. ax N*	0.0035649
	P. ax P myb-fl* vs. *P. ax P	**0.6321057**
	P. ax N vs. *P. ax P*	0.0095588
Stamen 5 length	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.0033786
	P. ax N myb-fl* vs. *P. ax N	**0.9999840**
	P. ax N myb-fl vs. *P. ax P*	0.0124603
	P. ax P myb-fl vs. *P. ax N*	0.0033526
	P. ax P myb-fl* vs. *P. ax P	**0.2928512**
	P. ax N vs. *P. ax P*	0.0123401
Corolla area	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.8994475
	P. ax N myb-fl* vs. *P. ax N	**0.8016682**
	P. ax N myb-fl vs. *P. ax P*	0.6277638
	P. ax P myb-fl vs. *P. ax N*	0.4941234
	P. ax P myb-fl* vs. *P. ax P	**0.8799663**
	P. ax N vs. *P. ax P*	0.3451968

Supplemental Table S3.4. Statistics of PTR-MS scent volatile measurements of *myb-fl* mutant lines. Statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons. Comparisons in bold font depict groupings of the *myb-fl* mutants with their respective wildtype lines.

Scent volatile	**Comparison**	**p-value** (adjusted with Tukey *post hoc* comparisons)
Methylbenzoate	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.6861634
	P. ax N myb-fl* vs. *P. ax N	**0.9950797**
	P. ax P myb-fl vs. *P. ax N*	0.8408502
	P. ax N myb-fl vs. *P. ax P*	0.7996604
	P. ax P myb-fl* vs. *P. ax P	**0.9871352**
	P. ax N vs. *P. ax P*	0.9282969
Benzaldehyde	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.0526532
	P. ax N myb-fl* vs. *P. ax N	**0.9999377**
	P. ax P myb-fl vs. *P. ax N*	0.0939637
	P. ax N myb-fl vs. *P. ax P*	0.0549258
	P. ax P myb-fl* vs. *P. ax P	**0.8952869**
	P. ax N vs. *P. ax P*	0.0984443
Iso/eugenol	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.3815730
	P. ax N myb-fl* vs. *P. ax N	**0.8942845**
	P. ax P myb-fl vs. *P. ax N*	0.9816308
	P. ax N myb-fl vs. *P. ax P*	0.6947641
	P. ax P myb-fl* vs. *P. ax P	**0.5312615**
	P. ax N vs. *P. ax P*	0.9854940

Supplemental Table S3.5. Statistics of pollen germination measurements of *myb-fl* mutant lines. Statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons. Comparisons in bold font depict groupings of the *myb-fl* mutants with their respective wildtype lines.

Measurement	**Comparison**	**p-value** (adjusted with Tukey *post hoc* comparisons)
Pollen germination	***P. ax N myb-fl* vs. *P. ax N***	**0.9806409**
	P. ax N vs. *P. ax P*	0.3939097
	P. ax P myb-fl vs. *P. ax N*	0.7671268
	P. ax N myb-fl vs. *P. ax P*	0.3084290
	P. ax N myb-fl vs. *P. ax P myb-fl*	0.9179023
	P. ax P myb-fl* vs. *P. ax P	**0.2052423**

Supplemental Table S3.6. Statistics of nectar concentration and volume measurements of *myb-fl* mutant lines. Statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons. Comparisons in bold font depict groupings of the *myb-fl* mutants with their respective wildtype lines.

Measurement	**Comparison**	**p-value** (adjusted with Tukey *post hoc* comparisons)
Nectar volume	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.0956810
	P. ax N vs. *P. ax P myb-fl*	0.1377268
	P. ax P* vs. *P. ax P myb-fl	**0.9575788**
	P. ax N myb-fl* vs. *P. ax N	**0.9456237**
	P. ax N myb-fl vs. *P. ax P*	0.2227059
	P. ax P vs. *P. ax N*	0.3158364
Nectar concentration	*P. ax N myb-fl* vs. *P. ax P myb-fl*	0.0122865
	P. ax N vs. *P. ax P myb-fl*	0.0163861
	P. ax P* vs. *P. ax P myb-fl	**0.7978693**
	P. ax N myb-fl* vs. *P. ax N	**0.8639512**
	P. ax N myb-fl vs. *P. ax P*	0.0332194
	P. ax P vs. *P. ax N*	0.0457742

Supplemental Table S3.7. RT-qPCR statistics of genes influencing flavonoid synthesis for *myb-fl* mutant lines (stage 4 bud limbs). Statistics were calculated for relative expression analyses of genes influencing flavonoid production in *P. axillaris* and *P. axillaris myb-fl* mutants (stage 4 bud limbs) obtained by RT-qPCR. As the data was not normally distributed, a Mann-Whitney U test was performed for each gene.

Gene	**W**	**p-value**
AN2	24	0.4418
MYB-FL	64	0.0001554
FLS	64	0.0001554
HT1 / F3'H	64	0.0001554
HF1 / F3'5'H	37	0.6454
HF2 / F3'5'H	29	0.7984
DFR	34	0.8785
ANS	32	1
GT3	34	0.5358
ART	36	0.7209
AAT	47	0.1304
GT5	31	0.9591
AMT3	30	0.8785
AMT35	29	0.7984
PH4	41	0.3823
DPL	38	0.5737

Supplemental Table S3.8. RT-qPCR statistics of transcription factors *MYB-FL* and *AN2* investigating spatial expression in *myb-fl* mutant lines. Statistics were calculated for relative expression analyses (stage 4 bud limbs, young leaves, pistils, sepals and stamens) obtained by RT-qPCR. As the data was not normally distributed, a Mann-Whitney U test was performed for each gene and sample type.

Gene	Sample type	W	p-value
AN2	floral limb	40	0.4418
	leaf	7	0.5556
	pistil	11	0.3929
	sepal	13	0.9143
	stamen	2	0.1429
MYB-FL	floral limb	0	0.0001554
	leaf	2	0.06349
	pistil	0	0.01587
	sepal	2	0.0381
	stamen	1	0.04762

Supplemental Table S3.9. Statistics of chlorophyll measurements in *myb-fl* mutant lines. Statistics were calculated for chlorophyll content analyses (SPAD values) in *P. axillaris* and *P. axillaris myb-fl* mutants. As the data was not normally distributed, a Mann-Whitney U test was performed for each comparison.

Comparison	W	p-value
P. ax myb-fl line 10 vs. WT line 10	76	0.1353
P. ax myb-fl line 3 vs. WT line 3	151	0.1149

Supplemental Table S3.10. Statistics of flavonoid measurements (spectrophotometer and HPLC) in *myb-fl* mutant lines. Statistics were calculated for flavonoid analyses (spectrophotometer and HPLC) in *P. axillaris* and *P. axillaris myb-fl* mutants. Spectrophotometer data was normally distributed and statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons. Statistics for HPLC data were calculated using a Mann-Whitney U test as data was not normally distributed.

Analysis	Flavonoids	Comparison	p-value
Spectrophotometer	Flavonols	*P. ax P myb-fl* vs. *P. ax N myb-fl*	0.5471
		P. ax N myb-fl vs. *P. ax N*	< 0.0001
		P. ax P myb-fl vs. *P. ax N*	< 0.0001
		P. ax N myb-fl vs. *P. ax P*	< 0.0001
		P. ax P myb-fl vs. *P. ax P*	< 0.0001
		P. ax P vs. *P. ax N*	0.0001
	Anthocyanidins	*P. ax P myb-fl* vs. *P. ax N myb-fl*	0.0003
		P. ax N myb-fl vs. *P. ax N*	< 0.0001
		P. ax P myb-fl vs. *P. ax N*	< 0.0001
		P. ax N myb-fl vs. *P. ax P*	< 0.0001
		P. ax P myb-fl vs. *P. ax P*	< 0.0001
		P. ax P vs. *P. ax N*	0.0907
HPLC	Delphinidin	*P. ax N myb-fl* vs. *P. ax N*	0.0009743
	Cyanidin	*P. ax N myb-fl* vs. *P. ax N*	0.0003305
	Quercetin	*P. ax N myb-fl* vs. *P. ax N*	0.7197

Supplemental Table S3.11. Hawkmoth (*M. sexta*) preferences for pairwise comparisons of *Petunia* species. Significant preference is indicated if $p < 0.05$.

Plant species 1	Plant species 2	First choice	Total choices	Hover	Probe	Forage
P. ax N	*P. inflata*	*P. ax N*	*P. ax N*	*P. ax N*	*P. ax N*	*P. ax N*
P. ax N	*P. secreta*	*P. ax N*	*P. ax N*	*P. ax N*	*P. ax N*	*P. ax N*
P. inflata	*P. secreta*	No pref.	*P. secreta*	*P. secreta*	*P. secreta*	*P. secreta*
P. ax N	*P. ax P*	No pref.	No pref.	No pref.	No pref.	No pref.
P. ax N	*P. ax N myb-fl*	*P. ax N*	*P. ax N*	*P. ax N*	*P. ax N*	*P. ax N*
P. secreta	*P. ax N myb-fl*	No pref.	*P. ax N myb-fl*	*P. ax N myb-fl*	*P. ax N myb-fl*	*P. ax N myb-fl*
P. ax P	*P. ax P myb-fl*	*P. ax P*	*P. ax P*	*P. ax P*	*P. ax P*	*P. ax P*
P. secreta	*P. ax P myb-fl*	*P. ax P myb-fl*	*P. ax P myb-fl*	No pref.	*P. ax P myb-fl*	No pref.
P. ax N myb-fl	*P. ax P myb-fl*	No pref.	*P. ax P myb-fl*	*P. ax P myb-fl*	*P. ax P myb-fl*	No pref.

Supplemental Table S3.12. Statistics for pairwise behavioral assays of *Petunia* species with hawkmoths (*M. sexta*). Data was analyzed using an exact binomial test. Differences were scored as significant if $p < 0.05$.

Plant comparisons		First choice			Total choices			Hover			Probe			Forage		
Species 1	Species 2	# of successes	# of trials	p-value	# of successes	# of trials	p-value	# of successes	# of trials	p-value	# of successes	# of trials	p-value	# of successes	# of trials	p-value
P. ax N	*P. inflata*	19	20	4.005e-05	262	298	< 2.2e-16	49	68	0.000358	112	123	< 2.2e-16	101	107	< 2.2e-16
P. ax N	*P. secreta*	19	20	4.005e-05	351	422	< 2.2e-16	107	126	4.524 e -16	155	172	< 2.2 e -16	89	124	1.339 e -06
P. inflata	*P. secreta*	6	20	0.1153	46	155	4.472e-07	14	48	0.005515	21	62	0.01513	11	45	0.0008241
P. ax N	*P. ax P*	8	20	0.5034	188	398	0.2925	73	161	0.2698	70	151	0.4159	45	86	0.7465
P. ax N	*P. ax N myb-fl*	2	20	0.0004025	349	412	< 2.2e-16	72	89	3.173 e -09	190	217	< 2.2 e -16	87	106	1.399 e -11
P. secreta	*P. ax N myb-fl*	14	20	0.1153	233	331	7.994e-14	72	105	0.0001784	90	118	8.914 e-09	71	108	0.001382
P. ax P	*P. ax P myb-fl*	4	21	0.007197	76	262	8.169e-12	22	80	7.011 e-05	33	114	8.044e-06	21	68	0.002186
P. secreta	*P. ax P myb-fl*	16	20	0.01182	108	163	4.014e-05	26	39	0.05325	53	77	0.001263	29	47	0.1439
P. ax N myb-fl	*P. ax P myb-fl*	6	20	0.1153	86	225	0.0004994	24	68	0.02053	43	112	0.01777	19	45	0.3713

Supplemental Table S3.13. Bumblebee (*B. terrestris*) preferences for pairwise comparisons of *Petunia* species. Significant preference is indicated if $p < 0.05$.

Plant species 1	***Plant species 2***	***First choice***	***Total choices***	***Touch***	***Visit***	***Forage***
P. ax N	*P. inflata*	*P. inflata*	*P. inflata*	*P. inflata*	*P. inflata*	*P. inflata*
P. ax N	*P. secreta*	No pref.	*P. secreta*	*P. secreta*	*P. secreta*	*P. secreta*
P. inflata	*P. secreta*	No pref.	*P. inflata*	No pref.	No pref.	*P. inflata*
P. ax N	*P. ax N myb-fl*	*P. ax N myb-fl*	*P. ax N myb-fl*	*P. ax N myb-fl*	*P. ax N myb-fl*	*P. ax N myb-fl*
P. secreta	*P. ax N myb-fl*	*P. secreta*	No pref.	No pref.	No pref.	No pref.
P. ax P	*P. ax P myb-fl*	No pref.	No pref.	No pref.	No pref.	No pref.
P. secreta	*P. ax P myb-fl*	*P. secreta*	*P. secreta*	No pref.	*P. secreta*	*P. secreta*
P. ax N myb-fl	*P. ax P myb-fl*	*P. ax N myb-fl*	*P. ax N myb-fl*	No pref.	No pref.	No pref.

Supplemental Table S3.14. Statistics for pairwise behavioral assays of *Petunia* species with bumblebees (*B. terrestris*). Data was analyzed using an exact binomial test. Differences were scored as significant if $p < 0.05$.

Plant comparisons		**First choice**			**Total choices**			**Touch**			**Visit**			**Forage**		
Species 1	Species 2	# of successes	# of trials	p-value	# of successes	# of trials	p-value	# of successes	# of trials	p-value	# of successes	# of trials	p-value	# of successes	# of trials	p-value
P. ax N	*P. inflata*	2	20	0.000402	8	117	< 2.2e-16	5	20	0.04139	2	23	6.604e-05	1	74	< 2.2e-16
P. ax N	*P. secreta*	7	23	0.09314	43	136	2.169e-05	35	99	0.004641	5	22	0.0169	1	11	0.01172
P. inflata	*P. secreta*	6	10	0.7539	41	62	0.01513	7	16	0.8036	7	13	1	27	33	0.0003241
P. ax N	*P. ax N myb-fl*	5	20	0.04139	5	42	4.434e-07	1	19	7.629e-05	4	17	0.04904	0	6	0.03125
P. secreta	*P. ax N myb-fl*	3	20	0.002577	29	47	0.1439	22	32	0.0501	7	14	1	0	1	1
P. ax P	*P. ax P myb-fl*	8	20	0.5034	10	23	0.6776	6	16	0.4545	4	7	1	0	0	1
P. secreta	*P. ax P myb-fl*	4	20	0.01182	37	46	4.056e-05	13	19	0.1671	14	16	0.004181	10	11	0.01172
P. ax N myb-fl	*P. ax P myb-fl*	15	20	0.04139	29	38	0.001658	15	21	0.07835	10	13	0.09229	4	4	0.125

Supplemental Table S3.15. Solitary bee (*O. cornuta*) preferences for pairwise comparisons of *Petunia* species. Significant preference is indicated if $p < 0.05$.

Plant species 1	***Plant species 2***	***First choice***	***Total choices***	***Touch***	***Visit***	***Forage***
P. ax N	*P. inflata*	*P. inflata*	*P. inflata*	*P. inflata*	No pref.	*P. inflata*
P. ax N	*P. secreta*	*P. secreta*	*P. secreta*	*P. secreta*	*P. secreta*	No pref.
P. inflata	*P. secreta*	No pref.	*P. inflata*	No pref.	No pref.	*P. inflata*
P. ax N	*P. ax N myb-fl*	No pref.	*P. ax N myb-fl*	No pref.	*P. ax N myb-fl*	No pref.
P. secreta	*P. ax N myb-fl*	No pref.	*P. sec*	*P. sec*	*P. sec*	No pref.
P. ax P	*P. ax P myb-fl*	No pref.	No pref.	No pref.	No pref.	No pref.
P. secreta	*P. ax P myb-fl*	*P. sec*	*P. sec*	*P. sec*	*P. sec*	No pref.
P. ax N myb-fl	*P. ax P myb-fl*	*P. ax N myb-fl*	*P. ax N myb-fl*	*P. ax N myb-fl*	*P. ax N myb-fl*	No pref.

Supplemental Table S3.16. Statistics for pairwise behavioral assays of *Petunia* species with solitary bees (*O. cornuta*). Data was analyzed using an exact binomial test. Differences were scored as significant if $p < 0.05$.

Plant comparisons		**First choice**			**Total choices**			**Touch**			**Visit**			**Forage**		
Species 1	Species 2	# of successes	# of trials	p-value	# of successes	# of trials	p-value	# of successes	# of trials	p-value	# of successes	# of trials	p-value	# of successes	# of trials	p-value
P. ax N	*P. inflata*	0	20	< 2.2e-16	1	100	< 2.2e-16	1	12	0.006348	0	5	0.0625	0	83	< 2.2e-16
P. ax N	*P. secreta*	1	20	4.005e-05	7	59	1.359e-09	5	39	2.43e-06	2	16	0.004181	0	4	0.125
P. inflata	*P. secreta*	14	20	0.1153	81	102	1.71e-09	7	19	0.3593	6	13	1	68	70	< 2.2e-16
P. ax N	*P. ax N myb-fl*	6	20	0.1153	7	33	0.001319	6	17	0.3323	1	11	0.01172	0	5	0.0625
P. secreta	*P. ax N myb-fl*	6	20	0.1153	45	55	2.057e-06	22	27	0.001514	18	22	0.004344	5	6	0.2188
P. ax P	*P. ax P myb-fl*	8	20	0.5034	13	27	1	8	15	1	5	11	1	0	1	1
P. secreta	*P. ax P myb-fl*	3	20	0.002577	66	80	3.137e-09	33	41	0.000112	22	25	0.0001565	11	14	0.05737
P. ax N myb-fl	*P. ax P myb-fl*	16	20	0.01182	32	38	2.434e-05	19	23	0.002599	8	9	0.03906	5	6	0.2188

Supplemental Table S3.17. Hawkmoth (*M. sexta*) preferences and statistics for pairwise comparisons of *Petunia* species with different light regimes. Data was analyzed using an exact binomial test. Differences were scored as significant if $p < 0.05$.

Plant comparisons		Light regime	First choice				Total choices			
Species 1	Species 2		Preference	# of successes	# of trials	p-value	Preference	# of successes	# of trials	p-value
P. ax P myb-fl	*P. ax P*	visible light, no UV	No preference	10	10	1	No preference	51	89	0.2031
P. ax N	*P. secreta*	no light	P. ax N	20	20	< 2.2e-16	P. ax N	91	98	< 2.2e-16

Supplemental Table S3.18. Number of no choices for each pollinator behavioral assay. No choices were recorded if a pollinator was observed flying in the flight arena but did not make a choice between any of the plants presented to them. Bumblebee pollinators had the highest number of no choices for all comparisons.

Experimental setup	Plant comparisons		Pollinators		
	Species 1	**Species 2**	**Hawkmoths (*M. sexta*)**	**Bumblebees (*B. terrestris*)**	**Solitary bees (*O. cornuta*)**
Natural *Petunia* species	*P. axillaris* N	*P. inflata*	1	13	3
Natural *Petunia* species	*P. axillaris* N	*P. secreta*	2	18	5
Natural *Petunia* species	*P. inflata*	*P. secreta*	1	19	1
myb-fl mutants	*P. axillaris* N	*P. axillaris* N *myb-fl*	1	27	1
myb-fl mutants	*P. secreta*	*P. axillaris* N *myb-fl*	0	14	3
myb-fl mutants	*P. axillaris* P	*P. axillaris* P *myb-fl*	0	14	5
myb-fl mutants	*P. secreta*	*P. axillaris* P *myb-fl*	0	10	3
myb-fl mutants	*P. axillaris* N *myb-fl*	*P. axillaris* P *myb-fl*	0	14	2
Light regime: visible light, no UV	*P. axillaris* P *myb-fl*	*P. axillaris* P	0	not tested	not tested
Light regime: no light	*P. axillaris* N	*P. secreta*	4	not tested	not tested

Supplemental Table S3.19. Primers for RT-qPCR, PCR and cloning experiments.

Gene	Application	Primer sequences 5' – 3'	Citation
AN2	RT-qPCR	AN2_q4_F: GCATTGAGAAGTATGGAGAAGG	Esfeld et al., 2018
		AN2_q4_R: TGTGGCCTTAGATAATTCAACC	
MYB-FL	RT-qPCR	MYB-FL_qPCR_F: TACCACCACCACTACCACAG	Sheehan et al., 2016
		MYB-FL_qPCR_R: ACCTATCGCTGCTCCTGCAT	
FLS	RT-qPCR	NA625: CCAAGTTGAGATTVTTAGCAATGG	Albert et al., 2014
		NA266: ACCGGCCATGACATTCTTG	
HT1 (F3'H)	RT-qPCR	B582: GACTTCCGCCATGTCCGC	Esfeld et al., 2018
		B583: GCACACGTTCAATAACTGGCC	
HF1 (F3'5'H)	RT-qPCR	B590: CGATATGAGTCGAGAGGGCC	Esfeld et al., 2018
		B591: TACCCTGCTATTGTCATTAACTCTACA	
HF2 (F3'5'H)	RT-qPCR	B596: GAAGTTTGGGAGAACCCACTAG	Esfeld et al., 2018
		B597: ACCATTACGATTCCCATTCTTGTC	
DFR	RT-qPCR	B578: AAGGCTGTCAAGGAGTATTTCATG	Esfeld et al., 2018
		B579: CCGGACTGTTGGCTTGATTAC	
ANS	RT-qPCR	B588: ATTTCTTCCATTGTGCTTTCCCTGA	Esfeld et al., 2018
		B589: ATCTGCTTGGCATATTCACTTGTTG	
3GT	RT-qPCR	B584: TCACAATCCAACACTTCAATCTTCTC	Esfeld et al., 2018
		B585: TAATAGCCTCAAGCCCAACTGG	
ART	RT-qPCR	B611: GCAATGCCAGCAGAGTCAAATCTATG	Esfeld et al., 2018
		B612: GTCTAAAGCAACCTTGAGAAGCTCAG	
AAT	RT-qPCR	B634: TCAGGACAGCTGAATTGCCTT	Esfeld et al., 2018
		B635: CGCAGCCGGAAAATCCATCA	
5GT	RT-qPCR	B632: TGAAAATGGACAGCCTATAACTTGCCTAC	Esfeld et al., 2018
		B633: GCCCGGGAAGTTGAATGGACC	
MT (3'AMT)	RT-qPCR	B613: AAGAGAGCATGAGCTACTCAAAGAAC	Esfeld et al., 2018
		B614: CATTTATGAGCTTTAGGAACATCGATAG	
MF (3'5'AMT)	RT-qPCR	B617: TGAAGCCCAATTTCTCTCGATGCTC	Esfeld et al., 2018
		B618: CAATCCAACCTCATATGCTTCTCTGTC	
PH4	RT-qPCR	B880: CATCATCATCATCAACAACAAGCAAC	Esfeld et al., 2018
		B881: ATTCTTTATCTCATTATCTGTTCTCCC	
DPL	RT-qPCR	B630: CTGAGGAAAGGAGCATGGG	Esfeld et al., 2018
		B879: GTATTCCAGTAGTTTTTCACATCG	
SAND	RT-qPCR	SAND_F: CTTACGACGAGTTCAGATGCC	Mallona et al., 2010
		SAND_R: TAAGTCCTCAACACGCATGC	
RAN1	RT-qPCR	RAN1_F: AAGCTCCCACCTGTCTGGAAA	Mallona et al., 2010
		RAN1_R: AACAGATTGCCGGAAGCCA	
ACTIN11	RT-qPCR	ACT11_F: TGCACTCCCACATGCTATCCT	Mallona et al., 2010
		ACT11_R: TCAGCCGAAGTGGTGAAAGAG	

Supplemental Table S3.20. Filtering for subgroup 7 MYBs. Differential expression in petal limbs (bud developmental stage 4) for the RNAseq dataset from *P. axillaris* and *P. axillaris myb-fl* mutants, filtered for the subgroup 7 R2R3-MYB transcription factors from Berardi et al., 2021.

Gene info			**Log2 fold change**		**Normalized read counts**	
Gene ID	Chromosome (*P. ax* v402)	*Petunia* gene name	L2FC[a]	padj L2FC	Average *axillaris*	Average *myb-fl*
Peaxi162Scf00001g00231	Chr6	NA	-0.13983811	0.96115841	4.607907999	5.000090081
Peaxi162Scf00147g00136	NA	NA	NA	NA	NA	NA

Chapter 4

Reconstructing pollination syndromes: mimicking a hawkmoth to bee transition

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Author contributions

Martina Lüthi: design of gRNA constructs for CRISPR/Cas9 gene editing; design of crossing scheme; analysis of *odo1* and *cnl1* mutant sequences; UV/VIS photography, nectar volume and concentration, pollen germination and morphology phenotypic measurements of *odo1* and *cnl1* mutants; analysis of phenotypic data (flavonoids, nectar, pollen, morphology); crossing of mutants; graphical visualization of all analyzed data; statistical analyses; writing of manuscript

Tracey Tenreira: design of gRNA constructs for CRISPR/Cas9 gene editing; genetic manipulation of *odo1* mutants; DNA extractions for sequencing of *odo1* mutants; analysis of *odo1* and *cnl1* mutant sequences; PTR-MS measurements of *odo1* and *cnl1* mutants

Lea Jäggi: genetic manipulation of *cnl1* mutants; DNA extractions for sequencing of *cnl1* mutants; spectrophotometer measurements of *odo1* and *cnl1* mutants

Loreta B. Freitas: provided plant materials

Cris Kuhlemeier: conceptualization; supervision

Abstract

Whether speciation occurs as a gradual process through many mutations of small effect or involves large mutational leaps in a few genes has been a longstanding debate. A system that allows speciation to be studied through large effect genes is pollinator-mediated speciation. The genus *Petunia* displays discrete pollination syndromes (floral traits associated with the attraction of specific pollinators) allowing the genetic changes underlying evolutionary shifts in pollinator attraction to be studied. These shifts in pollinators are complex and involve changes in a number of floral traits for which speciation genes have been described. Here we set out to reconstitute the evolutionary trajectory from hawkmoth to bee pollination by mutating the known speciation genes in a *P. axillaris* background (hawkmoth pollinated) to create an artificial mimic of *P. secreta* (bee pollinated). Through a CRISPR/Cas9 approach we targeted known speciation genes *ODO1* and *CNL1*, which control scent production through the benzenoid-phenylpropanoid pathway, disabling them through loss-of-function mutations. Benzenoid and phenylpropanoid production was eliminated in the *odo1* mutant line, while only benzenoid production was eliminated in the *cnl1* mutant line, essentially creating a scentless (*odo1*) and scent-reduced (*cnl1*) line. No other effects on phenotypic traits affecting pollinator preference were detected through these mutations. Combining these scent speciation genes with color speciation genes (*MYB-FL* and *AN2*) will now allow us to recreate the evolutionary events occurring during speciation in the lab, ultimately settling the longstanding debate about speciation occurring through few major effect mutations.

Introduction

The genetic basis of speciation continues to be one of the fundamental questions in biology. Darwin originally described speciation to be a gradual process by which populations entered new ecological niches and adapted to them, ultimately leading to the separation of two species (Darwin, 1859; Schluter, 2009). On a genetic basis this gradual process implies that many genetic changes of small effect slowly alter the phenotype. The opposing view is that speciation proceeds through mutations with a distribution of effect sizes, including both large and small effect mutations (Orr, 1998; Orteu and Jiggins, 2020). Orr proposed that mutations of large effect do not have to be deleterious to the speciation process as previously assumed, but can be beneficial (Orr, 1998). This theory has been demonstrated through an increasing amount of experimental evidence for such genes of large effects in both plants and animals (Ueshima and Asami, 2003; Doebley, 2004; Hoekstra et al., 2006; Chan et al., 2010; Nadeau et al., 2016; Todesco et al., 2020).

Both models are not mutually exclusive. For instance, in the case of allopatric speciation (when geographic isolation of populations occurs), many mutations of small effect may accumulate as the geographic barrier becomes more distinct. Through the geographical barrier, populations are separated and can no longer exchange genetic material leading to the cessation of gene flow (Coyne and Orr, 2004). The two populations can then diverge without interbreeding and speciation can take place through a gradual process. Although allopatry was long assumed to be the most common form of speciation, sympatric speciation (speciation occurring without geographical barriers) has been found to be the prevalent mode of speciation in more recent research (Schluter, 2009). In this scenario gradual genetic changes are less likely to be the genetic component of speciation as gene flow between the populations does not come to a halt. Based on theoretical work, having few mutations of large effect that quickly create large phenotypic differences between the populations can drive speciation when selection is strong (Orr and Coyne, 1992; Orr, 2005).

As we are forced to analyze the evolutionary trajectory in hindsight to find out how speciation has led to the species we can observe today, retracing the evolutionary steps of divergence is crucial to understanding the genetic basis of speciation. The higher the

number of mutations, the more difficult it becomes to retrace the steps as the number of possibilities becomes increasingly large. In species that have diverged through an infinite number of small effect mutations this process is genetically virtually intractable using current methods. Mutations of large effect have now been identified in a wide range of systems, offering compelling opportunities that allow these processes to be studied. Such genes of major effect that influence speciation have been coined speciation genes.

Rieseberg and Blackman (2010) define a speciation gene as a "gene that contributes to the splitting of two lineages by reducing the amount of gene flow between them". In other words, such genes need to contribute to isolation between species by influencing reproductive barriers. Isolation barriers act in a linear order, with prezygotic barriers occurring before postzygotic barriers. Due to this linearity, if multiple barriers are present, earlier barriers will contribute more to total isolation than later barriers. In flowering plants reproductive isolation is often caused by prezygotic isolation mechanisms as opposed to postzygotic ones (Ramsey et al., 2003; Rieseberg and Willis, 2007; Lowry et al., 2008; Widmer et al., 2009; Dell'Olivo et al., 2011). For genes to thus contribute to the speciation process in such systems they should most likely contribute to the reduction in gene flow through prezygotic barriers. Here we consider speciation genes to be genes whose contribution to reproductive isolation through prezygotic isolation barriers has been genetically and functionally validated.

A prime example of a system to study the process of speciation through such large effect mutations is pollinator-mediated speciation in plants, as selection on plants through pollinators is strong. Shifts in pollinators have happened frequently in the angiosperms and are widely accepted to be a driving force in their rapid diversification and speciation (Sapir and Armbruster, 2010; Schiestl and Johnson, 2013; Van der Niet et al., 2014). These shifts in pollinators are complex and involve changes in a number of floral traits that serve to attract pollinators and foster efficient pollen transfer. Further, pollinator attracting traits can be studied individually both on a genetic and phenotypic level, providing insight into the ongoing speciation process.

The South American genus *Petunia* (Solanaceae) has undergone recent diversification (approx. 2.85 – 1.3 million years ago) and consists of species with different pollination

syndromes, defined as suites of floral traits associated with attracting a specific pollinator (Lorenz-Lemke et al., 2006; Särkinen et al., 2013). This genus can be robustly separated into two clades: the short and long tube clade (Reck-Kortmann et al., 2014). The short tube clade includes the majority of *Petunia* species, which are all bee pollinated and phenotypically similar: purple, UV reflecting flowers with a short tube producing low amounts of scent (Reck-Kortmann et al., 2014; Amrad et al., 2016). The long tube clade, however, comprises species with different pollination syndromes. Species in this clade are hawkmoth, hummingbird or bee pollinated. All long tube species can be found in proximity to one another in southern Brazil.

P. axillaris and *P. secreta*, both species in the long tube clade, are morphologically nearly identical, but display differences in floral color and scent. *P. axillaris* has a white floral color, is UV absorbent and emits ample amounts of scent volatiles to attract nocturnal hawkmoths (Stehmann et al., 2009; Turchetto et al., 2015; Sheehan et al., 2016). *P. secreta* has purple colored flowers, is UV reflective and does not emit large amounts of scent volatiles (Stehmann and Semir, 2005; Rodrigues et al., 2018). Most recent phylogenetic studies based on both 400'000 SNPs and 984 amino acid sequences robustly place hawkmoth pollination as the ancestral state, with the bee pollinated *P. secreta* being a derived species (Esfeld et al., 2018). These different species in *Petunia* are of particular interest as multiple major effect genes responsible for phenotypic differences associated with pollinator attraction have been identified (Quattrocchio et al., 1999; Esfeld et al., 2018; Amrad et al., 2016; Sheehan et al., 2016). Particularly, floral color and scent, two major traits influencing the attraction of different pollinator types, are known to be encoded by speciation genes.

On a genetic level, the differences in floral traits between the long tube *Petunia* species are due to changes in four major effect genes: *AN2*, *MYB-FL*, *ODO1* and *CNL1* (Quattrocchio et al., 1999; Hoballah et al., 2007; Amrad et al., 2016; Sheehan et al., 2016; Esfeld et al., 2018). These genes are either involved in the regulation of floral color through the flavonoid biosynthetic pathway or scent volatile production through the benzenoid-phenylpropanoid pathway. *AN2* and *MYB-FL* encode R2R3-MYB transcription factors responsible for controlling the production of visible (anthocyanins) and UV (flavonols) color pigments respectively (Quattrocchio et al., 1999; Hoballah et al., 2007;

Sheehan et al., 2016; Esfeld et al., 2018). *ODO1* is also an R2R3-MYB transcription factor responsible for the regulation of scent volatile production located upstream of the shikimate pathway that feeds into the benzenoid-phenylpropanoid pathway (Verdonk et al., 2005; Klahre et al., 2011). In this same pathway *CNL1* encodes a CoA-ligase responsible for directing *t*-cinnamic acid into the beta-oxidative pathway that takes place in the peroxisomes activating the production of benzenoids (Klempien et al., 2012; Amrad et al., 2016). It is a combination of changes in floral color and scent through these speciation genes that has accompanied the shift from hawkmoth to bee syndrome in the long tube clade.

In the hawkmoth pollinated species *P. axillaris*, *AN2* is non-functional (Quattrocchio et al., 1999; Esfeld et al., 2018) while the other speciation genes are functional and either produce UV absorbing color compounds (*MYB-FL*) or scent volatiles (*ODO1* and *CNL1*) (Amrad et al., 2016; Sheehan et al., 2016). During the shift from hawkmoth to bee pollination, *P. secreta* has restored *AN2* function to produce purple color but has lost the ability to produce UV absorbing compounds due to low expression and a transposon insertion in *MYB-FL* (Esfeld et al., 2018). *P. secreta* has also lost its ability to produce scent volatiles, most likely through low expression of *ODO1* and *CNL1* (A. Berardi, unpublished data). As the speciation genes that lead to major phenotypic differences between *P. axillaris* and *P. secreta* have been identified, we can now use the differences in floral traits between these two species to mimic this pollination syndrome transition, essentially recreating evolution in a laboratory setting. Three specific phenotypic transitions need to be achieved to recreate this transition: a reduction or elimination of scent, a re-gain of visible color and a loss of UV color. Sequential mutation of the four speciation genes in an otherwise isogenic *P. axillaris* background will produce intermediate stages of this evolutionary process. Presenting these different combinations from single and double to triple mutants to pollinators for behavioral assays should reveal how the magnitude of phenotypic effects influences primary and secondary pollinator preference.

Here we set out to reconstitute the evolutionary trajectory from hawkmoth to bee pollination by mutating the known speciation genes in a *P. axillaris* background in such a way as to create a *P. secreta* mimic. Testing pollinator preference should help answer important questions regarding the level of reproductive isolation and thereby the

speciation process in *Petunia*. How many genes are needed to obtain speciation? Are all four speciation genes needed to mimic *P. secreta* or are some phenotypically redundant? How do these genes affect pollinator preference individually and in combinations? How much additional variation will remain after mutating the known speciation genes? Answering these questions would allow us to retrace the evolutionary steps and help resolve the phylogeny in the recently diverged long tube clade. Ultimately this should settle the longstanding debate about the contributions of infinite mutations of small phenotypic effect versus few mutations of large effect. In this study we used a CRISPR/Cas9 approach to mutationally inactivate the scent speciation genes *ODO1* and *CNL1* in *P. axillaris*. We then combined these mutated scent genes with the inactivated color speciation genes *MYB-FL* and reactivated *AN2* (see Chapter 2 and 3) all in an isogenic *P. axillaris* background to determine the individual and combined effects of these major effect genes on speciation in *Petunia*.

Materials and methods

Plant material

P. axillaris ssp. axillaris P (hereafter referred to as *P. axillaris* P) originates from the University of Bern Botanical Garden (Hoballah et al., 2007). *P. secreta* was collected in its natural habitat Galpão de Pedra, Rio Grande do Sul, Brazil and maintained by selfing. All plants were grown in commercial soil (70% Klasman substrate, 15% Seramis clay granules, 15% quartz sand) under a light:dark regime of 15:9 h at 22:17 °C in a growth chamber. Plants were fertilized once a week with a nitrogen-phosphorous-potassium and iron fertilizer.

Color and UV image scoring

UV images were recorded with a converted Nikon D7000 SLR camera and Nikon 60 mm 2.8D microlens (aperture F9, ISO 800, shutter speed 1/10 s). The camera was converted to block visible and infrared light, allowing UV light to be recorded using a UV-specific filter (Advanced Camera Services). UV-A light (320-390 nm) was provided as a light source when capturing images through a modified Metz MZ76 flash gun (Advanced Camera Services). Using Photoshop CS4 (Adobe Systems) all images were converted to grayscale. Exposure compensation across the entire image was adjusted depending on

the species. Comparison with wildtype *P. axillaris* P flowers, which are completely absorbent, allowed scoring as either UV reflective or UV absorbent. Color images were recorded with a Canon EOS 60D camera and Canon 35 mm lens (aperture F4.5 – F7.0, ISO 100 – 200, shutter speed 1/30 s). All images were captured under identical incandescent lighting and with a black background for standardized comparison with a color calibration card (X-Rite ColorChecker Classic Mini)

Floral scent volatile analysis

Methylbenzoate, benzaldehyde, isoeugenol and eugenol compounds were analyzed as described previously in Klahre et al., 2011 and Amrad et al., 2016 using a proton transfer reaction mass spectrometry (PTR-MS) approach. Flowers were collected one day post-anthesis right before the onset of dark in the growth chamber (15:9 h light:dark cycle at 22:17 °C). Scent volatiles of all plants were measured for 20 cycles. Five flowers per plant were analyzed as biological replicates. Samples were tested for their significance using a one-way ANOVA with Tukey *post hoc* comparisons in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463 as data was normally distributed. Isoeugenol and eugenol are isomers and thus cannot be separated via PTR-MS and were therefore analyzed as a single compound.

Measurement of additional floral traits

Corolla surface size and morphological traits (style and pistil lengths) were analyzed using front and side view photographs. For morphological traits, flowers were dissected to reveal reproductive organs inside the floral tube. Stamens were numbered according to their placement and size inside the floral tube (1 - 5 ranging from longest stamen to shortest in clockwise order around the floral axis); *Petunia* stamens are arranged in two different sizes (4+1) (Stehmann and Semir, 2005). All pictures were analyzed using the ImageJ software to extract lengths and corolla areas of the samples. Five flowers per plant were analyzed as biological replicates. Nectar volume and concentration were measured according to the protocol described in Brandenburg et al., 2012. All flowers were sampled right before the onset of dark in the growth chamber to account for any fluctuations in nectar production during the day:night cycle. Five flowers per plant were analyzed as biological replicates. All measured traits were tested for their significance using a one-

way ANOVA with Tukey *post hoc* comparisons in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463 as data was normally distributed.

Pollen germination measurements

After 2h incubation in germination medium, pollen germination rates were determined. Germination medium consisted of a 10x salt solution (10 mM $CaCl_2$, 10 mM KCl, 8mM $MgSO_4$ x 7 H_2O, 16 mM boric acid, 300 µM $CuSO_4$ x 5 H_2O; salt solution end concentration 1x), 1.5% Casein hydrolysate (end concentration 0.03%), sucrose (end concentration 5%), MES monohydrate (end concentration 15 mM) and PEG 6000 (end concentration 12.5%). The pH value of the solution was adjusted to 5.8 with 1 M KOH and filter sterilized. Single freshly dehisced anthers were dipped into 300 µl germination medium and incubated at room temperature for 2 h. Care was taken to not shake samples after incubation to avoid damaging pollen tubes that had grown. Samples were prepared on microscopy slides after incubation and the percentage of germinated pollen grains was determined. Samples were tested for their significance using a one-way ANOVA with Tukey *post hoc* comparisons of normally distributed data in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463.

Spectrophotometric quantifications of floral flavonols and anthocyanidins

Total flavonol and anthocyanidin (anthocyanin aglycone) absorbance values were measured for all flower petal limb samples using an Ultraspec 3100 pro (GE Healthcare Life Sciences) spectrophotometer, scanning from 365 nm to 530 nm. Three discs (8 mm diameter) were punched out of the floral limb tissue and placed in 1 ml of 2N HCl to soak for 15 min at room temperature (22 °C), hydrolyzed at 100 °C for 15 min, cooled for 10 min at 4 °C, then centrifuged for 3 min at 14000 rpm. Supernatants were removed and analyzed directly to avoid degradation of pigments. Absorbance values for each sample were measured at 530 nm and 365 nm (Harborne, 1998). Five flowers were sampled per plant to include technical replicates for each biological replicate (individual plant). As data was not normally distributed pairwise comparisons of samples were performed using a Mann-Whitney U test in R v3.6.1 (R Core Team, 2017) and RStudio v1.3.463.

Generating CRISPR constructs

gRNAs targeting exons 1 and 2 of the *ODO1* and *CNL1* genes were designed and checked for off-target effects using the CRISPOR tool (Concordet and Haeussler, 2018). Double gRNA constructs under the tomato U6 promoter and *A. thaliana* U6-26 promoter (Genbank X51447.1 and KY080693.1) containing attL sites for Gateway cloning were ordered from Genscript (New Jersey, USA). These constructs were then cloned into pDECas9-Kan destination vectors using the LR reaction from the Gateway cloning protocol (Thermo Fisher). Final destination vectors were amplified in *E. coli* and then purified using the extraAxon plasmid mini kit (Axonlab). The destination vectors were then transformed into *A. tumefaciens* strains GV3101 and LBA4404 and grown on solid selective medium at 28 °C for 2 days before being used for subsequent stable plant transformation.

Stable transformation of *P. axillaris* P

P. axillaris P leaves were transformed using *A. tumefaciens* (strains GV3101 or LBA4404) carrying either the *ODO1* or *CNL1* CRISPR construct. *A. tumefaciens* cultures were grown for 2 days on solid media (YEB media with appropriate antibiotics Rifampicin, Streptomycin, Spectinomycin or Gentamycin) at 28 °C before inoculating 5 mL YEB liquid medium (with appropriate antibiotics Streptomycin, Spectinomycin or Gentamycin) with *A. tumefaciens* cultures to grow overnight at 28 °C. Liquid overnight cultures were then centrifuged at 3500 rpm for 15 min, the liquid supernatant discarded and the pellet resuspended in 40 ml of autoclaved MilliQ water with 0.01 mM acetosyringone to aid *A. tumefaciens* infection. Liquid cultures were kept in the dark at room temperature until used for plant inoculation. Leaves from both *P. axillaris* species (from 4-6 week old plants) were sterilized in a 10% hypochlorite solution for 10 min and rinsed five times with autoclaved MilliQ water. The sterilized leaves were then cut into 1 cm^2 pieces and inoculated in the *A. tumefaciens* suspension for 30 min (in the dark with shaking). The leaf fragments were dried between two layers of sterilized filter paper and transferred to solid Murashige-Skoog (MS) growth medium without antibiotic selection. Leaf pieces were grown in the dark for 7 days at a constant temperature of 24 °C. After this first week, leaf pieces were then transferred to fresh selective growth medium containing Kanamycin to select for the presence of the CRISPR construct. All plates were sealed with medical tape and incubated under the same temperature conditions and a light:dark

regime of 16:8 h. Leaf fragments were transferred to fresh media once a week or more often if contaminations were detected for callus growth to be successful. Once shoots started to appear, each shoot was excised from the calli and transferred to MS rooting medium containing kanamycin for further selection of the construct. This allowed roots to form and shoots were transferred to soil (70% Klasman substrate, 15% Seramis clay granules, 15% quartz sand) when their roots reach a length of 2-3 cm. Plants were then grown in the same growth chamber and under the same conditions as all other species (see section plant material for details). Screening for CRISPR mutations was then performed on these plants after one week to allow them to acclimatize to the soil conditions.

DNA extractions and genotyping

Genomic DNA extractions were performed with fresh leaf samples using a modified CTAB (Murray and Thompson, 1980) or SDS protocol. Briefly samples were ground in 200 µl SDS extraction buffer, centrifuged at maximum speed for 10 min. The supernatant was then removed and mixed with 100 µl iso-propanol. After 10 min incubation, samples were centrifuged again at maximum speed for 10 min before the supernatant was discarded. Samples were washed with 100 µl 75% ethanol (centrifuged at maximum speed for 5 min), dried overnight and then resuspended with 100 µl water. Samples were analyzed with a Nanodrop ND-1000 (Thermo Fisher) prior to further analysis. Target sequences were amplified using the GoTaq (Promega) polymerase with primers depicted in Suppl. Table S4.6. PCR reactions were run according to the manufacturer's protocol with the following parameters for *ODO1*: annealing temperature 58 °C, elongation time 2:00 min with 35 cycles and *CNL1*: annealing temperature 56 °C, elongation time 1:00 min with 35 cycles. Sequences were obtained through Sanger sequencing (GATC, Cologne, Germany and Microsynth, Balgach, Switzerland) with the primers listed in S4.6. Sequence confirmation for samples of interest were obtained through cloning (pGEM-T Easy kit, Promega) and subsequent Sanger sequencing. Purification of PCR samples prior to sequencing was done using the NucleoFast 96 PCR plate (Macherey-Nagel). All *ODO1* and *CNL1* sequences were analyzed manually using the Geneious Primer 2020.2.4 software (Biomatters). Sequences of transformed plants were aligned to existing wildtype sequences of *P. axillaris* P accessions.

Combining mutants and pyramiding traits

Using our previously developed color mutants (*AN2* expression and *myb-fl*, Chapter 2 and 3 respectively) and combining them with our scent mutants (*odo1* and *cnl1*), we used a crossing scheme to stack these traits. Crosses and selfings were alternated between generations to obtain homozygous lines of single, double and triple mutants. All mutants were developed in a hawkmoth pollinated *P. axillaris* P background in order to mimic the transition to the bee pollinated *P. secreta* plant in the long tube clade.

Results

Mutations of major effect genes via CRISPR/Cas9

To mimic the transition from hawkmoth to bee pollination, we aimed to mutate each known speciation gene involved in pollinator preference in *Petunia*. As pollinators use multiple floral cues, such as color and scent, to distinguish between plant species, we targeted the two described scent speciation genes, *ODO1* and *CNL1*, involved in the benzenoid-phenylpropanoid scent pathway in *Petunia* (Amrad et al., 2016) to combine these with our mutated color genes (as described in Chapters 2 and 3). Using the newly adapted CRISPR/Cas9 protocol we previously developed for wild *Petunia* species (see Chapter 2), we transformed *P. axillaris* P lines. Both genes were targeted with a double gRNA approach. gRNAs were designed to target the first two exons of each gene respectively (Fig. 4.1). Two independent mutant lines were developed for each gene, with different sizes of mutations. The *ODO1* gene was mutated in exon 1 and exon 2 through a 1 bp insertion in both sites (Fig. 4.1 A). Although a double gRNA was used to target the *ODO1* gene, mutations in both gRNAs were never observed simultaneously. Contrary to this, the *CNL1* gene showed mutations in both gRNAs at the same time (Fig. 4.1 B). While insertions were observed in the *ODO1* gene, deletions of different lengths were found in the *CNL1* gene. All mutations in the *ODO1* and *CNL1* genes led to premature stop codons, resulting in truncated proteins. Mutations of the *CNL1* gene in exons 1 and 2 truncated the AMP-dependent synthase/ligase domain that contains the conserved AMP-binding site while mutations in exon 1 of *ODO1* truncated R2 and R3 domains.

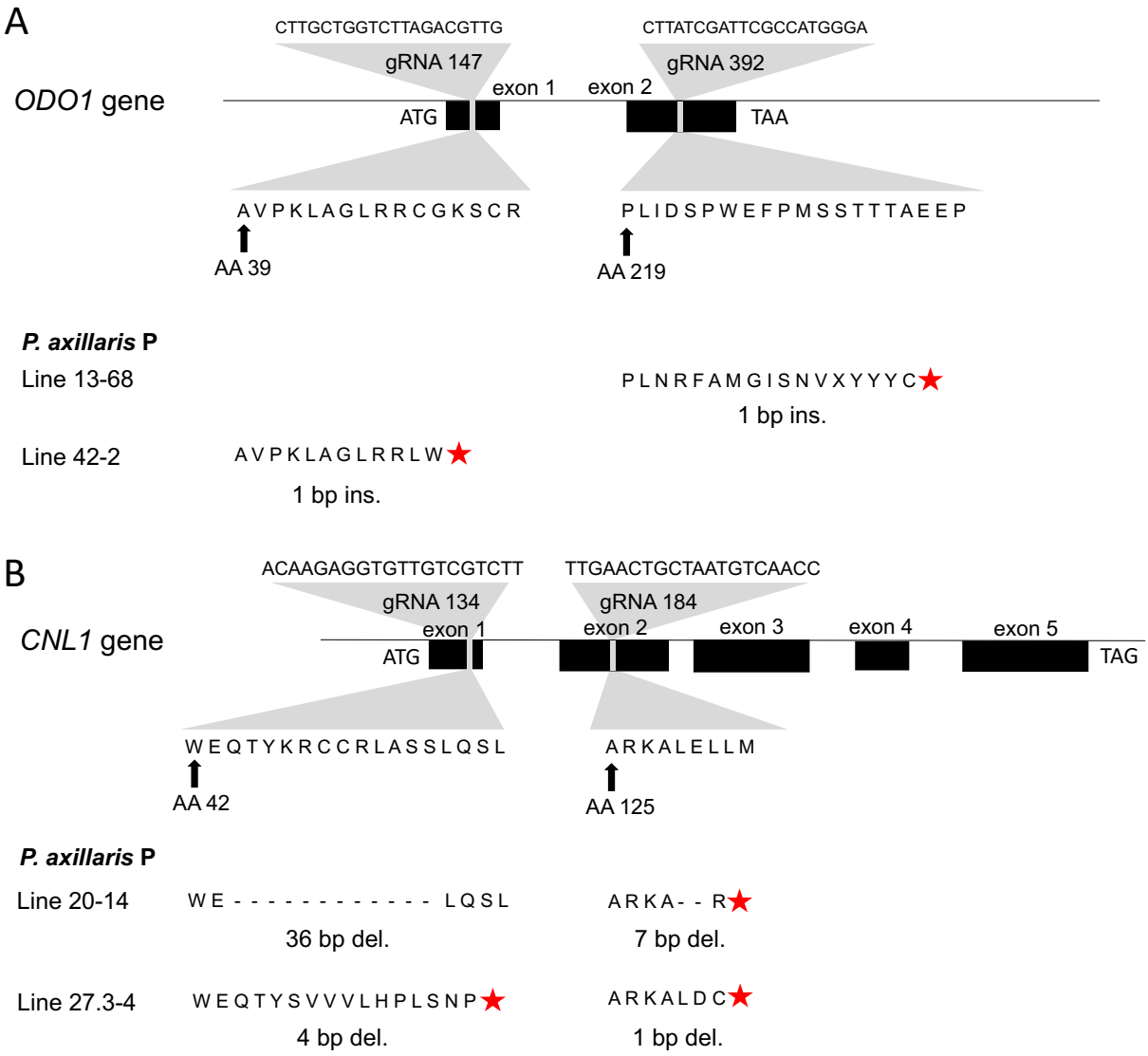

Figure 4.1: Mutations in *ODO1* and *CNL1* genes using a CRISPR/Cas9 approach. A double gRNA CRISPR/Cas9 construct was used to target exons 1 and 2 of the *ODO1* (A) and *CNL1* (B) genes in *P. axillaris* P. Two independent mutant lines of each species were generated through insertions and deletions all leading to truncated proteins through a premature stop codon.

Strong phenotypic changes in scent emission due to CRISPR/Cas9-mediated knock-out of *ODO1* and *CNL1*

To characterize the phenotypic aspects of the *ODO1* and *CNL1* knockout on pollination syndrome traits, we analyzed the scent profile of the *P. axillaris* P mutant lines. As *ODO1* is an activating transcription factor of the benzenoid-phenylpropanoid biosynthetic pathway, we predicted that a non-functional protein would lead to a scentless phenotype. The position of *CNL1* downstream in the pathway, after the branch producing isoeugenol/eugenol has split off (Fig. 1.6, Chapter 1), led us to hypothesize that these

compounds would not be altered by a mutation in the *CNL1* gene. The emission at dusk of the benzenoids methylbenzoate and benzaldehyde, main compounds eliciting responses from hawkmoths in electroantennogram studies (Hoballah et al., 2005; Klahre et al., 2011) as well as a the phenylpropanoids isoeugenol and eugenol, that also elicit hawkmoth electroantennogram responses (Hoballah et al., 2005), was measured for the two independent *odo1* and *cnl1* mutant lines compared to *P. axillaris* P wildtype lines. Low (< 5 ppb) to no scent volatiles were detected in the *odo1* mutants (Fig. 4.2, full statistics in Suppl. Table S4.1). The *cnl1* mutant showed low to no methylbenzoate and benzaldehyde emission, but maintained the same levels of isoeugenol/eugenol emission as in the wildtype (Fig. 4.2). Our phenotypic analyses of the *odo1* and *cnl1* mutants demonstrated that a knockout of *odo1* was enough to eliminate scent in the mutant lines, while mutating *cnl1* altered scent volatile composition by causing a reduction in benzenoid emission (methylbenzoate and benzaldehyde compounds), but not altering phenylpropanoid (isoeugenol/eugenol) emission.

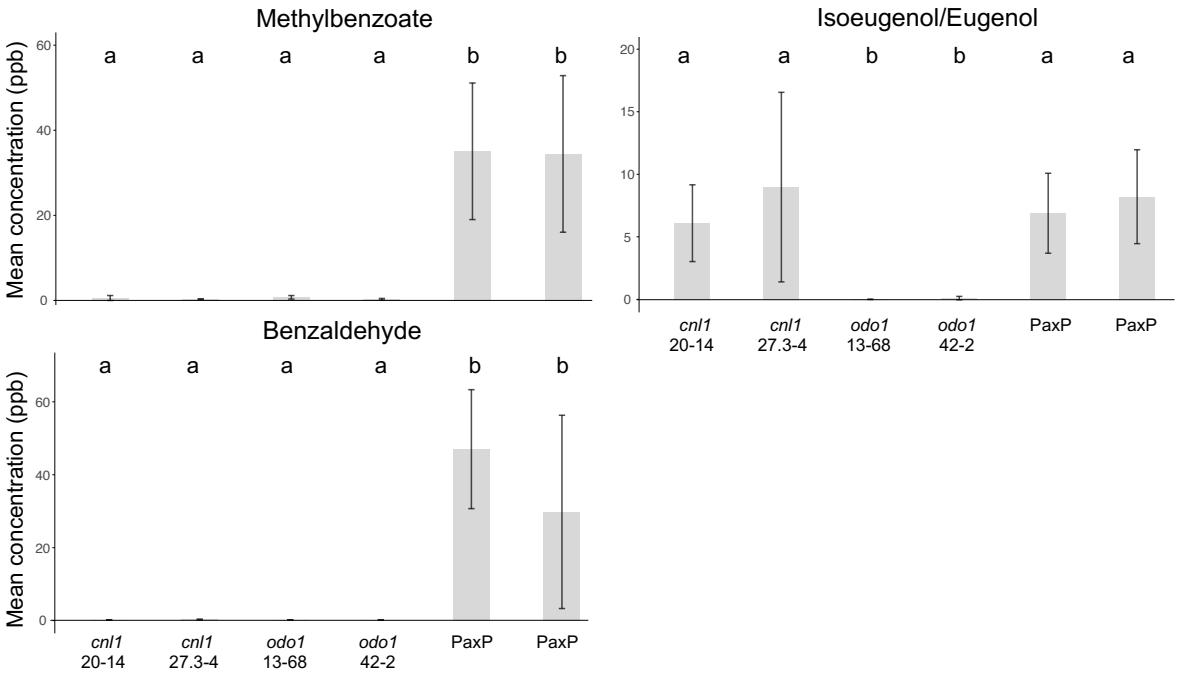

Figure 4.2: Scent volatile analysis of *odo1* and *cnl1* mutants. Two independent transformation lines per targeted gene were measured via PTR-MS. The three compounds methylbenzoate, benzaldehyde and isoeugenol/eugenol, representing benzenoids and phenylpropanoids, of the scent volatile profile were compared. Bars show mean values of 5 measured flowers per plant ± SD. Statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons. Significant differences between groups are indicated with letters (full statistics in Suppl. Table S4.1).

Knock-out of *odo1* and *cnl1* does not affect the visible and UV color phenotype

The transcription factor *ODO1* activates the shikimate pathway (Fig. 1.6, Chapter 1) that leads into the phenylalanine biosynthesis pathway responsible for the production of phenylalanine, which is simultaneously a precursor for production of scent volatiles (benzenoids, phenylpropanoids) as well as color pigments (anthocyanins, flavonols) (Amrad et al., 2016; Patrick et al., 2021). It was therefore important to quantify floral color phenotype in the scent mutant lines. We did not find any phenotypic differences in floral color phenotype. Both mutants were UV absorbent and white-colored, like the wildtype *P. axillaris* P phenotype (Fig. 4.3 B). We measured these samples through UV-VIS spectrophotometric analysis to quantify flavonol and anthocyanidin absorbance. There were no significant differences in flavonol absorbance in either the *odo1* or *cnl1* mutant compared to the *P. axillaris* P wildtype (Fig. 4.3 A, full statistics in Suppl. Table S4.2). The same was observed for anthocyanidin absorbance: no differences were detected in the *odo1* and *cnl1* mutants (Fig. 4.3 A). Anthocyanidin absorbance was low (< 0.05) in all samples and led to the visible colorless phenotype in the mutants (Fig. 4.3 A). This also correlates with the *myb-fl* mutants described in Chapter 3 where mean absorbance values needed to be > 0.05 to be able to induce faint pink coloration in the *myb-fl* mutants visible through VIS photography. Even though *ODO1* is a transcription factor that acts upstream of both the benzenoid-phenylpropanoid and flavonoid pathway and activates the shikimate pathway that leads into the phenylalanine biosynthesis pathway producing the precursor phenylalanine for color and scent production, it does not affect the floral color phenotype, but displays a scent pathway specific alteration in phenotype.

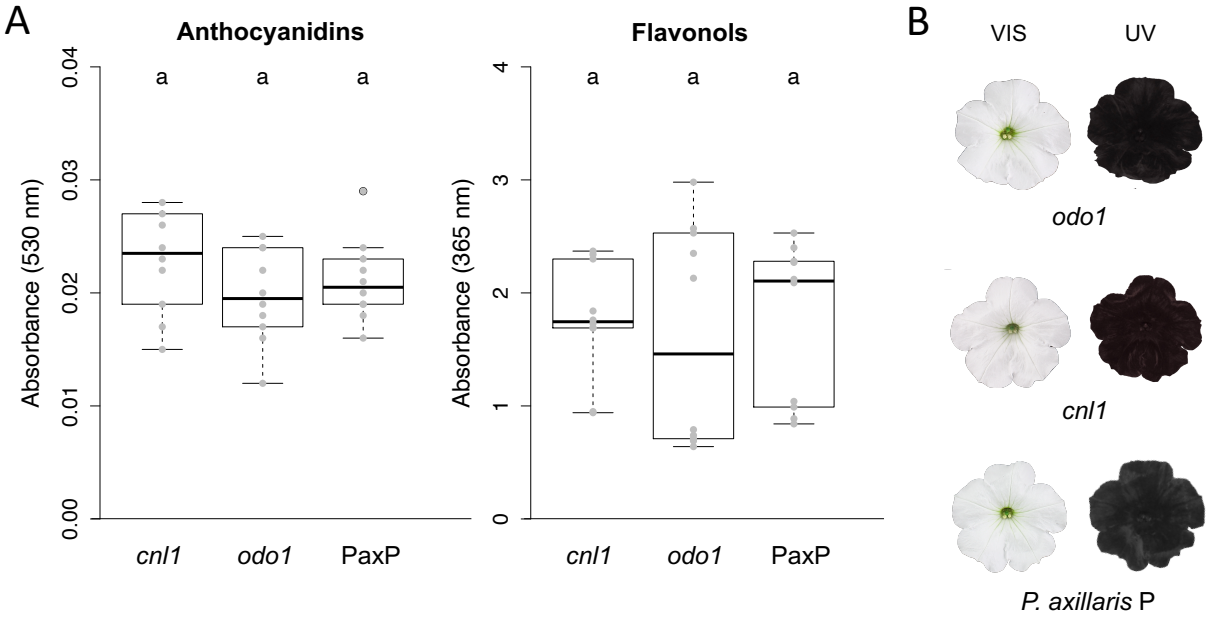

Figure 4.3: **Flavonoid absorbance in *odo1* and *cnl1* mutant lines.** (A) Flavonol and anthocyanidin absorbance of *odo1* and *cnl1* mutant lines compared to *P. axillaris* P as determined through UV-VIS spectrophotometric analysis. Letters display significant differences calculated for pairwise comparisons using a Mann-Whitney U test. Full statistics are shown in Suppl. Table S4.2. (B) Visible and UV color photos of *odo1* and *cnl1* mutant lines compared to the *P. axillaris* P wildtype. No visible differences in color phenotype were detected.

Phenotypic effects of *odo1* and *cnl1* mutations are specific to scent pathway

To further determine whether the *odo1* and *cnl1* mutations had an effect on other phenotypic traits that could potentially affect pollinator preference, we also assessed corolla size, pistil and stamen length, nectar volume and concentration, as well as pollen germination in the scent mutants. No further differences between wildtype and mutant lines were found in any of the additionally measured pollination syndrome phenotypic traits. Nectar volume and sugar concentration of nectar did not vary between the scent mutants and wildtype lines (Fig. 4.4 A-B, full statistics in Suppl. Table S4.3). Pollen germination rates were also not significantly different in any of the mutant lines (Fig. 4.4 C, full statistics in Suppl. Table S4.4). Morphological traits, such as corolla area and length of pistils and stamens, were also not affected by the *odo1* and *cnl1* mutations (Fig. 4.4 D-E, full statistics in Suppl. Table S4.5). Overall the mutations in both of these scent genes elicit very specific phenotypic responses restricted only to the production of scent volatiles in *Petunia*.

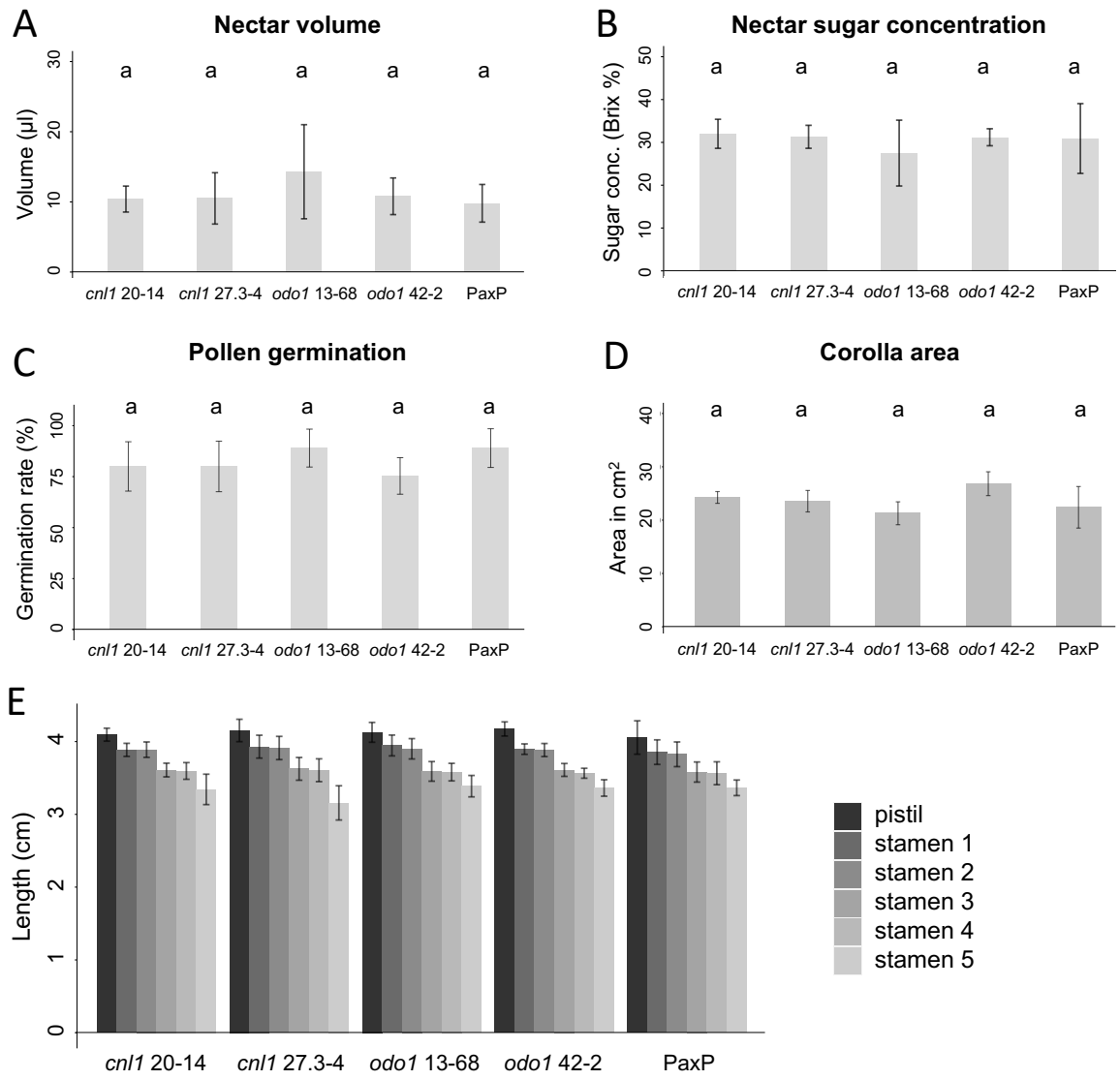

Figure 4.4: **Phenotypic measurements of scent mutant lines**. *Odo1* and *cnl1* mutants (2 independent mutant lines per gene) were compared to wildtype *P. axillaris* P. All bars depict mean values of 5 measured flowers per plant ± SD. Statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons. Significant differences between groups are indicated with letters (full statistics in Suppl. Tables S4.3-S4.5). (A) Nectar volume and (B) sugar concentration in the different samples is depicted. (C) Pollen germination rates were determined 2 h post inoculation in germination medium. (D) Corolla areas for mutant and wildtype lines. (E) Pistil and stamen lengths were measured individually for each pistil and five stamens per flower.

Combining traits to create a *P. secreta* mimic plant

In order to mimic the transition from a hawkmoth to bee pollination syndrome in *Petunia*, we aimed to combine the mutated genes through a crossing scheme (Fig. 4.5). Since our phenotypic analyses of the *odo1* and *cnl1* mutants demonstrated that a knockout of *odo1* was enough to eliminate scent in the mutant lines, adding an additional *cnl1* knockout to

the *odo1* line should not further alter the scent phenotype for pollinators. We therefore aim to produce two parallel mimic lines; a completely scentless line with a non-functional *odo1* and a scent reduced line with a non-functional *cnl1* (Fig. 4.5, Table 4.1) controlling for possible interacting effects of the mutations through phenotypic analyses in every generation.

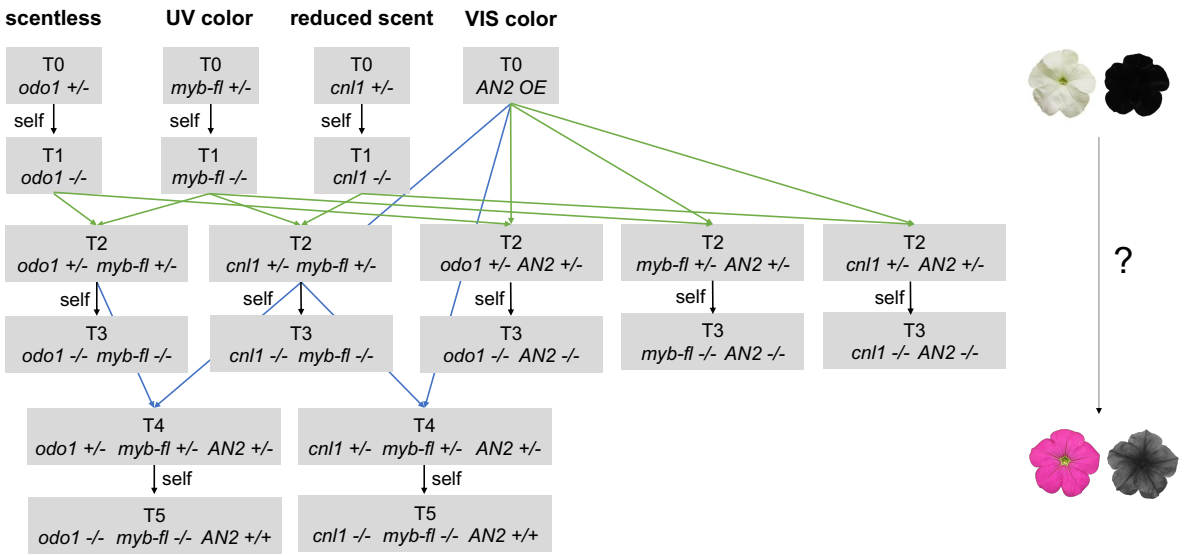

Figure 4.5: Crossing scheme for creating a *P. secreta* mimic plant. Two parallel lines were developed with the two scent speciation genes (*ODO1* and *CNL1*). To obtain homozygous mutants, selfs and crosses were alternated in the crossing scheme (black arrows = selfing, green arrows = crosses for obtaining double mutants, blue arrows = crosses for obtaining triple mutants). Final mimic lines contain three mutated speciation genes each to alter floral UV/VIS color and scent volatiles. The entire crossing scheme was done with two independent mutant lines per gene.

Table 4.1. Predicted phenotypes of mutants developed with crossing scheme. Different combinations of phenotypic traits are expected for single, double and triple mutant lines.

Generation	Mutant line	Phenotype		
		Visible color	**UV color**	**Scent**
T1 single mutants	*myb-fl*	faint pink	UV reflective	scented
	AN2	pink	UV reflective	scented
	odo1	white	UV absorbent	scentless
	cnl1	white	UV absorbent	scent reduced
T3 double mutants	*myb-fl / odo1*	faint pink	UV reflective	scentless
	myb-fl / cnl1	faint pink	UV reflective	scent reduced
	myb-fl / AN2	pink (darker?)	UV reflective	scented
	AN2 / odo1	pink	UV absorbent	scentless
	AN2 / cnl1	pink	UV absorbent	scent reduced
T5 triple mutants	*myb-fl / AN2 / odo1*	pink (darker?)	UV reflective	scentless
	myb-fl / AN2 / cnl1	pink (darker?)	UV reflective	scent reduced

Discussion

To mimic the shift from hawkmoth to bee pollination syndrome in *Petunia,* three traits important for pollinator attraction (visible color, UV color and scent production) need to be altered. As the hawkmoth and bee pollinated species mainly differ in floral scent and color, for which speciation genes have been described (Quattrocchio et al., 1999; Hoballah et al., 2007; Amrad et al., 2016; Sheehan et al., 2016), we were able to specifically alter scent production in *P. axillaris* by targeting the *ODO1* and *CNL1* genes and can now combine these with mutations in the scent genes *MYB-FL* and *AN2*.

Complete elimination of scent through mutation of a single transcription factor

The transcription factor *ODO1*, that we targeted via CRISPR/Cas9, is upstream of the shikimate pathway, that leads into the benzenoid-phenylpropanoid pathway, responsible for production of scent volatiles (Amrad et al., 2016). We therefore expected that a knock-out of this transcription factor would result in a downregulation of all scent volatiles

involved in pollinator attraction (Fig. 4.2). The mutation of MYB transcription factors seems to be favored as evolutionary targets (Yuan et al., 2013). However, knocking out a transcription factor that functions early in the pathway increases the risk of pleiotropic effects as *ODO1* interacts with other regulatory factors in complex feedback loops (Verdonk et al., 2005; Spitzer-Rimon et al., 2010, 2013). For example, the shikimate pathway leads into the phenylalanine biosynthesis pathway producing the precursor phenylalanine for floral pigmentation (Patrick et al., 2021), which could be affected by a non-functional *ODO1*. Although both color and scent production rely on the precursor phenylalanine, we were not able to detect any pleiotropic effects on floral color (Fig. 4.3). Color and scent biosynthesis do not occur during the same developmental stages: flavonoid biosynthesis occurs during the bud stage while scent production occurs in the open flower stage (Amrad et al., 2016; Sheehan et al., 2016). Temporal separation of color and scent biosynthesis during flower development is therefore most likely responsible for the specific effects on scent production we observed in the *odo1* mutants.

Targeting *CNL1* specifically alters scent composition

Targeting *CNL1* instead of *ODO1* elicited a more specific phenotypic response on scent compound composition (Fig. 4.2 and 4.3) and is less likely to induce pleiotropic effects. *CNL1* catalyzes the production of the C_6-C_1 volatiles in the benzenoid-phenylpropanoid pathway. Especially hawkmoths strongly respond to volatiles produced through this branch of the pathway, such as methylbenzoate and benzaldehyde (Hoballah et al., 2005). Both the *odo1* and *cnl1* mutants no longer produced the benzenoid scent volatiles methylbenzoate and benzaldehyde, while the *cnl1* mutant still produced the phenylpropanoid isoeugenol/eugenol compounds (Fig. 4.2). Our *cnl1* mutant therefore may appear scentless to hawkmoths but could still attract other pollinators through a different composition of scent volatiles. However, hawkmoths also react to the C_6-C_3 compounds isoeugenol/eugenol, although not as strongly as to the C_6-C_1 compounds (Hoballah et al., 2005). It is therefore possible that subtle differences in pollinator preference may be detected between the scentless (*odo1*) and scent reduced (*cnl1*) lines. Even if such subtle differences were detected, they may not influence plant reproduction since certain hawkmoth pollinated species, such as *P. axillaris subsp. parodii,* do not produce any isoeugenol/eugenol in nature (Koeduka et al., 2009; Klahre et al., 2011). The *cnl1* mutation could also confer negative fitness effects, as not only pollinators are

attracted by scent volatiles, but other unwanted visitors, such as herbivores, as well (Theis, 2006; Theis and Adler, 2012; Kessler et al., 2013). The scentless *odo1* mutants could therefore show an increased survival rate compared to the scent reduced *cnl1* mutants if exposed to their natural herbivore community.

Studying the effects of the scentless and scent reduced mutations on secondary pollinator behavior (bee pollination) will determine whether these subtle differences in scent emission played a role during the transition from hawkmoth to bee pollination syndrome. Bees are able to discriminate between scent emission patterns, guiding them towards floral rewards (Skaliter et al., 2021). The highest internal pool levels of scent volatiles in *Petunia* are found in the floral limb (Verdonk et al., 2003), where bees land during pollination. A gradient of scent volatiles along the floral limb allows bees to discriminate between the petal rim and base of *Petunia* corollas (Skaliter et al., 2021). Since bees are sensitive to changes in scent emission on such a small scale, they may exhibit stronger response differences between the *odo1* and *cnl1* mutants than the hawkmoths.

Determining the number of major effect genes necessary for the transition from hawkmoth to bee pollination syndrome

Our crossing scheme (Fig. 4.5) aims to determine whether altering a few genes of major effect is enough to create reproductive isolation between two *Petunia* species. This will settle the longstanding debate on whether speciation can occur through few large mutational leaps. As *ODO1* and *CNL1* have been described to be scent speciation genes and *AN2* and *MYB-FL* to be color speciation genes, we aim to combine all four genes to create a complete *P. secreta* mimic. From a phenotypic point of view, including knock-outs of both *CNL1* and *ODO1* in a single plant, should not alter pollinator behavior when compared to the single *ODO1* mutant as they are phenotypically redundant. This phenotypic redundancy raises the question of whether mutating only three speciation genes is enough for reproductive isolation through pollinators to occur between *P. axillaris* and our *P. secreta* mimic. Additionally we observed that loss of UV color led to a higher amount of anthocyanidins (Chapter 3) while mutating *ODO1* also led to an alteration in amounts of anthocyanidins, albeit at a level so low that no phenotypic differences were observed (Fig. 4.3). Interactions between these mutated speciation

genes could therefore either intensify or diminish the observed phenotypes and will provide further information on the number of genes needed for reproductive isolation.

To ideally determine precisely how many genes of large phenotypic effect are needed to recreate the hawkmoth to bee pollination syndrome transition in a laboratory setting, we would need to use the common ancestor of the long tube clade that the species have developed from. As this common ancestor no longer exists, we can recreate this speciation event with *P. axillaris*, the species that is the closest to what we predict the ancestor to have looked like. Potential residual variation in our *P. secreta* mimic compared to the *P. secreta* wildtype is plausible due to two reasons: (1) Divergence of *P. axillaris* after it's separation from *P. secreta*, leading to degradation of non-functional genes or (2) Changes in a few major effect genes account for the majority of phenotypic changes needed to shift from hawkmoth to bee syndrome, but some additional fine-tuning through genes of small effect may be needed to eliminate residual variation. This potential residual variation could therefore help us determine even more precisely what percentage of phenotypic trait differences are due to major effect genes.

Pollinators will determine a successful plant mimic

To test if we were able to mimic the hawkmoth to bee pollinator transition through the mimic plants produced in our crossing scheme, testing the plants with both pollinator types is key. In the single mutant lines, *myb-fl* mutants were tested with both pollinators (Chapter 3) and represented an intermediate stage of the mimic process based on pollinator preference. Hawkmoths significantly preferred *P. axillaris* P over the *myb-fl* mutant while bees still preferred *P. secreta* compared to the mutant (Fig. 4.6 A). When presented with conflicting cues (scent and color) hawkmoths are able to use both cues equivalently (Klahre et al., 2011). We therefore predict the single *odo1* mutants to have equally strong effects on hawkmoth pollinator preference as the *myb-fl* mutants (Fig. 4.6 A). The *AN2* and *cnl1* mutants should also elicit a pollinator response but it will most likely be weaker than for the *myb-fl* and *odo1* mutants as their phenotypic effects are not as strong. The *AN2* mutant only alters visible color (Chapter 2) compared to the *myb-fl* mutant where UV and visible color was altered (Chapter 3) while the *cnl1* mutant still produces the phenylpropanoids isoeugenol/eugenol and therefore is not completely scentless as opposed to the *odo1* mutant. When combining these traits, if the transition

has been successfully mimicked, both pollinator types should not be able to distinguish between the mimic and *P. secreta* plants when presented with the two (Fig. 4.6 B). Using these different assays should allow us to evaluate how strongly the mutations in the individual genes cause a shift in pollinator preference.

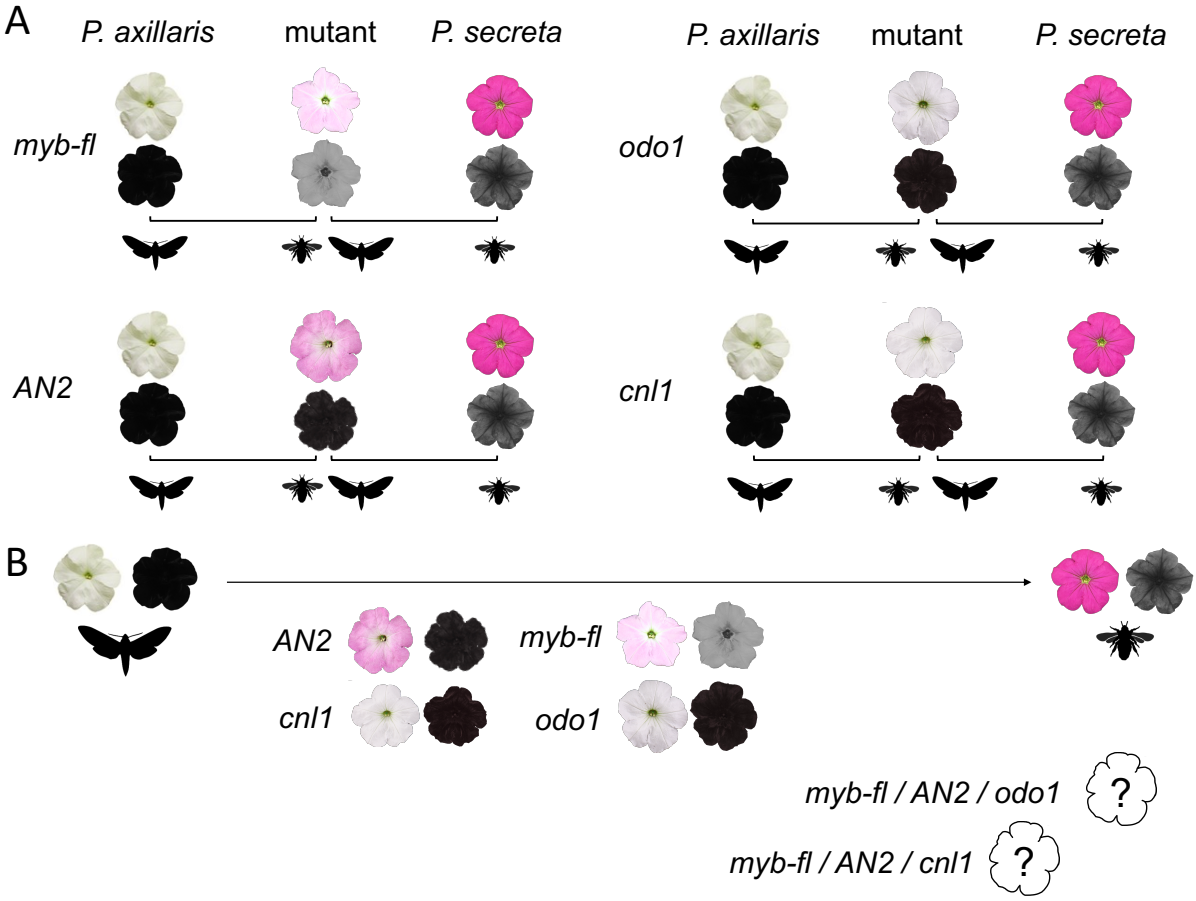

Figure 4.6: **Shifts in pollinator preference based on single speciation gene mutations or combinations of mutated speciation genes.** (A) Predicted pollinator choices for the four single mutants compared to *P. axillaris* or *P. secreta* wildtypes, depicted by the axes below each comparison. Pollinator preferences for *myb-fl* mutants have been verified (Chapter 3) while preferences for *AN2, odo1* and *cnl1* mutants are based on predictions. Positions of the pollinators along the axis between a species comparison displays their predicted or verified preference. (B) Predicted positions of intermediate phenotypes of mutant lines along the hawkmoth to bee transition scale. The left hand side of scale represents a flower adapted to hawkmoth pollination while the right end represents bee pollination. The position of the mutants along the axis demonstrates their predicted stage along the hawkmoth to bee transition. Single gene *myb-fl* mutants display an intermediate phenotype in the shift from hawkmoth to bee syndrome and the same is predicted for *odo1* mutants. The other two single gene mutants *AN2* and *cnl1* are expected to elicit a weaker shift in pollinator response, as their phenotypic effects are not as strong as the effects of the other two genes. Reactivating *AN2* only altered visible color while mutating *myb-fl* altered UV and visible color simultaneously. The *odo1* mutation created a scentless phenotype while there are still scent volatiles produced in the *cnl1* mutant. Triple mutants (depicted as floral outlines as the phenotype has not yet been

determined) should then move the mimic plant even closer to the *P. secreta* phenotype and ideally no longer evoke differential pollinator responses between the mimic plant and *P. secreta*.

Retracing the order of events in a shift from hawkmoth to bee pollination syndrome

Recent radiations, as is the case in *Petunia*, are often difficult to resolve due to ongoing gene flow, standing genetic variation and incomplete lineage sorting (Edwards et al., 2016; Mallet et al., 2016). For instance the phylogenetic history of the classical model system *Mimulus* that has been used for decades to study the genetics of speciation has recently been overturned (Nelson et al., 2021). Using a whole-genome species tree hummingbird pollination is now suggested to have a single origin no longer supporting the sister status of the bee pollinated *M. lewisii* and the hummingbird pollinated *M. cardinalis* (Nelson et al., 2021). Discordance between the gene trees seems to be caused through introgression between species. This example demonstrates the need for analyses on both a single gene and genome-wide level to determine phylogenetic relationships and speciation processes. Phylogenetic reconstruction of the *Petunia* genus on a genome-wide scale has robustly placed hawkmoth pollination as the ancestral state in the long tube clade (Esfeld et al., 2018). By reconstructing the genetic events causing shifts in pollination syndromes in mimic plants we can now test these shifts experimentally in functional pollinator assays, retracing the evolutionary events occurring during the transition from hawkmoth to bee syndrome. Combining our mimic approach with the reconstructed phylogeny and molecular studies that have identified the causative mutations involved in pollination syndrome shifts, could resolve uncertainties in classical phylogenies and help create a comprehensive model of speciation.

Currently the order of steps leading away from hawkmoth pollination syndrome has been tentatively suggested to be a loss of scent followed by a loss of UV color and finally a gain of visible color (Amrad et al., 2016). The *CNL1* gene is more strongly degenerated than the genes responsible for color, which is why it was most likely mutated earlier during the speciation process (Amrad et al., 2016). The shift towards visible color is estimated to have happened more recently than the loss of UV color, as a single causal mutation in the *MYB-FL* gene can be found in almost all UV absorbing accessions, while multiple independent mutational events can be found in the *AN2* gene (Sheehan et al., 2016; Esfeld

et al., 2018). This is supported by our *myb-fl* mutants (see Chapter 3) where the trade-off between flavonol and anthocyanin production, puts a constraint on the order of color changes. For visible color to be restored, flavonol production must first be reduced to free up precursors for the production of anthocyanins. If these molecular analyses of the gene in natural populations and mutant lines can now be coupled with pollinator assays of all individual combinations of scent and color traits determining how strong isolation is for each combination, this could help retrace the process of speciation. Exploiting this speciation gene system in *Petunia* would make this the first study to recreate the entire process of speciation during the shift from hawkmoth to bee syndrome, directly demonstrating speciation through few major effect genes.

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Supplemental data

Supplemental Table S4.1. Statistics for PTR-MS measurements of scent volatiles in *odo1* and *cnl1* mutants (flowers one day post anthesis). Statistics were calculated for *P. axillaris*, *P. axillaris odo1* and *P. axillaris cnl1* mutants using a one-way ANOVA with Tukey *post hoc* comparisons for each combination of mutant and wildtype line.

Scent volatile	**Comparison**	**p-value** (adjusted with Tukey *post hoc* comparisons)
Methylbenzoate	*P. ax P odo1* vs. *P. ax P cnl1*	0.9864134
	P. ax P vs. *P. ax P cnl1*	< 0.0000001
	P. ax P vs. *P. ax P odo1*	< 0.0000001
Benzaldehyde	*P. ax P odo1* vs. *P. ax P cnl1*	0.9999637
	P. ax P vs. *P. ax P cnl1*	0.0247941
	P. ax P vs. *P. ax P odo1*	0.0246928
Isoeugenol / Eugenol	*P. ax P odo1* vs. *P. ax P cnl1*	0.0209761
	P. ax P vs. *P. ax P cnl1*	0.9999326
	P. ax P vs. *P. ax P odo1*	0.0208652

Supplemental Table S4.2. Statistics of flavonoid measurements (spectrophotometer) for *odo1* and *cnl1* mutant lines. Flowers were sampled one day post anthesis in *P. axillaris* wildtype lines as well as *P. axillaris odo1* and *cnl1* mutants. As the data was not normally distributed, a Mann-Whitney U test was performed for each comparison.

Flavonoids	**Comparison**	**W**	**p-value**
Flavonols	*P. ax P odo1* vs. WT	44	0.7
Anthocyanidins	*P. ax P odo1* vs. WT	43	0.6
Flavonols	*P. ax P cnl1* vs. WT	48	0.9
Anthocyanidins	*P. ax P cnl1* vs. WT	64	0.3
Flavonols	*P. ax P odo1* vs. *P. ax P cnl1*	54	0.8
Anthocyanidins	*P. ax P odo1* vs. *P. ax P cnl1*	70	0.1

Supplemental Table S4.3. Statistics of nectar concentration and volume measurements in *odo1* and *cnl1* mutant lines. Statistics for flowers one day post anthesis in *P. axillaris*, *P. axillaris odo1* and *P. axillaris cnl1* mutants were calculated using a one-way ANOVA with Tukey *post hoc* comparisons for each combination of mutant and wildtype line.

Measurement	**Comparison**	**p-value** (adjusted with Tukey *post hoc* comparisons)
Nectar volume	*P. ax P odo1* vs. *P. ax P cnl1*	0.5584562
	P. ax P vs. *P. ax P cnl1*	0.9517943
	P. ax P vs. *P. ax P odo1*	0.5252980
Nectar concentration	*P. ax P odo1* vs. *P. ax P cnl1*	0.5494656
	P. ax P vs. *P. ax P cnl1*	0.9449147
	P. ax P vs. *P. ax P odo1*	0.8008200

Supplemental Table S4.4. Statistics of pollen germination measurements of *odo1* and *cnl1* mutant lines. Statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons for each combination of mutant and wildtype line.

Measurement	**Comparison**	**p-value** (adjusted with Tukey *post hoc* comparisons)
Pollen germination	*P. ax P odo1* vs. *P. ax P cnl1*	0.9475028
	P. ax P vs. *P. ax P cnl1*	0.6113476
	P. ax P vs. *P. ax P odo1*	0.7316314

Supplemental Table S4.5. Statistics of phenotypic measurements of organ lengths and corolla area for *odo1* and *cnl1* mutant lines (flowers one day post anthesis). Statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons for each combination of mutant and wildtype line.

Organ	**Comparison**	**p-value** (adjusted with Tukey *post hoc* comparisons)
Pistil length	*P. ax P odo1* vs. *P. ax P cnl1*	0.7810948
	P. ax P vs. *P. ax P cnl1*	0.4478467
	P. ax P vs. *P. ax P odo1*	0.2962577
Stamen 1 length	*P. ax P odo1* vs. *P. ax P cnl1*	0.9180959
	P. ax P vs. *P. ax P cnl1*	0.5168913
	P. ax P vs. *P. ax P odo1*	0.4059096
Stamen 2 length	*P. ax P odo1* vs. *P. ax P cnl1*	0.8501397
	P. ax P vs. *P. ax P cnl1*	0.0884995
	P. ax P vs. *P. ax P odo1*	0.1084222
Stamen 3 length	*P. ax P odo1* vs. *P. ax P cnl1*	0.5134573
	P. ax P vs. *P. ax P cnl1*	0.2599126
	P. ax P vs. *P. ax P odo1*	0.5556274
Stamen 4 length	*P. ax P odo1* vs. *P. ax P cnl1*	0.1555364
	P. ax P vs. *P. ax P cnl1*	0.1455921
	P. ax P vs. *P. ax P odo1*	0.7863470
Stamen 5 length	*P. ax P odo1* vs. *P. ax P cnl1*	0.5064106
	P. ax P vs. *P. ax P cnl1*	0.6454642
	P. ax P vs. *P. ax P odo1*	0.9957332
Corolla area	*P. ax P odo1* vs. *P. ax P cnl1*	0.9982806
	P. ax P vs. *P. ax P cnl1*	0.9041450
	P. ax P vs. *P. ax P odo1*	0.8855786

Supplemental Table S4.6. Primers for PCR and cloning experiments.

Gene	**Application**	**Primer sequences 5' – 3'**	**Citation**
ODO1	PCR	BT003_F: GCCATACACTTCCCATTAATCA	
		BT004_R: ATCCGTGCCTGTTCTCTACG	
CNL1	PCR	cdsCNL_F: ATGGACGAGTTACCAAAATGTGG	Amrad et al., 2016
		B811_R: CTCGTCGTCAACAATATTCTCGG	

Chapter 5

General Discussion

Mutations in single genes confer major phenotypic changes affecting reproductive isolation

The overarching goal of this thesis was to target single genes of major phenotypic effect, coined "speciation genes", on a molecular level and test these with pollinator behavioral assays to determine their individual contributions to reproductive isolation. I previously defined speciation genes as genes contributing to isolation between species by influencing reproductive barriers for which substantial work to fulfill at least two of the requirements on functional, organismal and population-level studies outlined by Rieseberg and Blackman (2010) had been done (Chapter 1). One of the most important types of reproductive barriers is animal-mediated pollination. As pollinators are directly linked to the plant's reproductive success, they can exert selection on floral traits, such as color, shape and scent. Depending on the pollinator assemblage these selection pressures can diverge, resulting in phenotypic differences in floral traits. Specific suites of floral traits, termed pollination syndromes, have evolved to attract a specific group of pollinators. Here I investigated changes in both UV and visible floral color and how these influence the behavior of different pollinator guilds. Effects on primary and secondary pollinators were determined by using *Petunia* species with two different pollination syndromes, the hawkmoth pollinated *P. axillaris* and the bee-pollinated *P. secreta*. Transitions between these species have involved changes in both UV and visible color caused by differences in flavonols and anthocyanins, as well as scent production through volatiles that are emitted. Previous work had shown that four major speciation genes are responsible for these shifts in color and scent: *MYB-FL* for UV color, *AN2* for visible color, *ODO1* and *CNL1* for scent production (Quattrocchio et al., 1999; Hoballah et al., 2007; Klahre et al., 2011; Amrad et al., 2016; Sheehan et al., 2016; Esfeld et al., 2018). The identification of these important genes offered a unique opportunity to target and recreate the evolutionary events that have happened during pollinator shifts in the long tube clade in *Petunia*, in a laboratory setting.

To mimic the transition from a hawkmoth to bee pollination syndrome in *Petunia*, I knocked out the speciation gene *MYB-FL* (Chapter 3) in a *P. axillaris* background through CRISPR/Cas9 mutations leading to strong specific phenotypic changes without the occurrence of other unpredicted pleiotropic effects. Pollinator behavioral assays on *myb-fl* mutants showed strong shifts in primary pollinator preference (hawkmoths) indicating a strong impact on reproductive isolation between *Petunia* species based on this single gene (Chapter 3). While primary pollinators significantly preferred the wildtype *P. axillaris* species, secondary pollinators (bumblebees and solitary bees) were also able to distinguish between the wildtype and *myb-fl* mutants and displayed opposite preference compared to the hawkmoths. I aimed to combine *MYB-FL* with further mutated speciation genes *ODO1* and *CNL1* (Chapter 4) in a *P. axillaris* background, to incorporate the loss of scent that have occurred during the transition from hawkmoth to bee pollination syndrome. Similar strong behavioral differences in pollinator preference for the speciation genes *ODO1* and *CNL1*, involved in scent production, are expected as scent and color cues have been shown to be equally important to attract insect pollinators (Klahre et al., 2011). While the transition from a hawkmoth to bee pollination syndrome involved the loss of scent and UV color, but the gain of visible color, I also aimed to restore function of the *AN2* gene. Through an overexpression approach with strong, petal-specific *CHS-A* promoters, I was able to restore color in a *P. axillaris* background (Chapter 2). However, color was not restored to the same extent as can be found in *P. secreta* but would still most likely induce shifts in pollinator behavior. Collectively the data presented in this thesis reveals that reproductive isolation can indeed be caused through mutations in single genes of large phenotypic effect and that mutating a single gene, *MYB-FL,* is enough to cause a strong shift in pollinator preference.

Visible color restoration in *Petunia* is constrained

There is only a single known event of restoration of the *AN2* gene in the long tube clade that has led to a gain in visible color (Esfeld et al., 2018), demonstrating that this event seems to be a very special case of gene resurrection. A possible explanation for why changes in transcription factor *AN2* are uncommon is because it requires both bHLH and WD40 partners to activate target promoters and induce transcription (Koes et al., 2005; Albert et al., 2011). In contrast *MYB-FL* homologs in *Arabidopsis* do not require a bHLH

factor to activate targets (Zimmermann et al., 2004; Mehrtens et al., 2005). If *AN2* must interact with two partners to activate transcription while *MYB-FL* does not, this could explain why complete shifts in flavonol accumulation (to UV absorbing or reflecting flowers) are easier to achieve than restoring anthocyanin accumulation. However, it must also be considered that the production of flavonols and anthocyanins happens through the same biosynthetic pathway and their individual levels of accumulation may be linked.

A trade-off between flavonols and anthocyanins determines floral color in *Petunia*

Comparing the visible color phenotypes of the *AN2* transgenic lines (Chapter 2) with the *myb-fl* mutant lines (Chapter 3), gives insight into the flavonol/anthocyanin trade-off in the flavonoid biosynthetic pathway. The negative association between anthocyanins and flavonols has also been observed in UV reflective sectors of F1 individuals (*P. axillaris* dTph4 transposon line x *P. exserta*) containing a higher amount of anthocyanins than the UV absorbent sectors (Sheehan et al., 2016). The most likely hypothesis for these results is substrate competition for the dihydroflavonol precursors that both types of compounds are derived from. FLS and DFR are the two enzymes competing directly for the same precursors and responsible for the anthocyanin and flavonol branches of the pathway. In our RNAseq experiment we observed that through mutation and subsequent downregulation of *MYB-FL*, *FLS* was significantly downregulated and thus directly activated by *MYB-FL* (Fig. 3.5 Chapter 3). *DFR*, the anthocyanin-branch counterpart to *FLS*, was not significantly upregulated, demonstrating that *MYB-FL* does not seem to repress this gene. Therefore, in wildtype *P. axillaris*, highly expressed *FLS* seems to out-compete lowly expressed *DFR* for substrate (Fig. 3.5, Chapter 3). However, by mutating *MYB-FL* the competition for precursors is shifted in favor of the anthocyanin producing branch by reduction of *FLS* expression. To determine the phenotype of *Petunia* lines with both a non-functional *MYB-FL* and a functional *AN2* through the nature of this trade-off, it will be interesting to analyze lines where we have combined the *myb-fl* mutant with our *AN2* overexpression lines. If this competition for substrates is as strong as we have predicted, the anthocyanin accumulation in these combined lines should be enhanced.

Substrate competition has not only been detected in *P. axillaris* as I present here, but also in other *Petunia* lines, such as the *Petunia* Mitchell cultivar. Inhibition of *FLS* production in this *Petunia* line resulted in an increase in anthocyanin production and a decrease in flavonol levels (Davies et al., 2003). This pattern is not restricted to the *Petunia* genus but has also been observed in further systems as well. In *Mimulus* the R2R3-MYB transcription factor *LAR1* (*LIGHT AREAS1*) regulates expression of *FLS* and belongs to the same subgroup of R2R3-MYB transcription factors (SG7) as *MYB-FL* (Yuan et al., 2016). The up- or downregulation of *FLS*, depending on the allelic status of *LAR1*, correlates with the presence/absence patterning of white areas on the pink colored corolla as the metabolic flux in the anthocyanin biosynthetic pathway is either pushed towards the colorless flavonols or colored anthocyanins (Yuan et al., 2016). Examining the *FLS* and *DFR* genes from various species (*Rosa rugosa*, *Prunus persica* and *Petunia hybrida*) revealed the same trend, where competition for common substrates directs biosynthesis towards different branches of the pathway (Luo et al., 2016). This was also observed in *Arabidopsis*, where *myb11 myb12 myb111* mutants led to decreased amounts of flavonols but an increase in anthocyanin accumulation (Stracke et al., 2007).

It is also possible that this trade-off between flavonols and anthocyanins is present but has not yet been detected in other species. Since visible color changes can be detected by the human eye, unpredicted alterations will most likely be investigated even if this is not the scope of a study. However, changes in UV color and flavonol accumulation may not be detected as they are not visible to the human eye, suggesting an observation bias. Additionally phenotypic analyses of UV color are more challenging to perform as specific equipment (e.g. UV camera) or biochemical analyses are needed. As determining changes in UV color becomes easier with enhanced equipment, detection of trade-offs between the anthocyanin and flavonol branches of the flavonoid biosynthetic pathway could become more frequent in other plant systems.

R2R3-MYB transcription factors as targets of evolution

In many cases involving transitions in floral color or scent, R2R3-MYB transcription factors have been found to be crucial (Hoballah et al., 2007; Lowry et al., 2012; Quattrocchio et al., 1999; Schwinn et al., 2006; Shang et al., 2011; Streisfeld et al., 2013;

Yuan et al., 2013; Sheehan et al., 2016; Esfeld et al., 2018; Lin and Rausher, 2021). Of the four speciation genes discussed in this thesis, three belong to this gene family (*MYB-FL*, *AN2* and *ODO1*). This suggests that, in *Petunia*, R2R3-MYB transcription factors are preferentially targeted during speciation as opposed to genes of other families that could confer similar effects. Even though phenotypes can be altered by directly targeting biosynthetic genes (Coberly and Rausher, 2003; Zufall, 2003; Fehr and Rausher, 2004; Smith et al., 2008; Smith and Rausher, 2011) these changes may be irreversible and possibly deleterious. It has also been demonstrated that spontaneous mutation rates do not seem to be higher in the R2R3-MYB family than in other classes of genes (Streisfeld and Rausher, 2011). If this is the case, why are R2R3-MYB transcription factors a preferred target during evolution?

One possible explanation is the developmental and tissue specificity of MYBs, detailed by my observations for the *MYB-FL* R2R3-MYB transcription factor (Chapter 3). Mutating *MYB-FL* caused few very specific phenotypic differences, most likely due to the high degree of spatial and developmental specificity in floral color gene expression. We observed differential regulation of only a few genes of the flavonoid pathway and alteration of UV color (and anthocyanidins to a lesser extent) as a direct effect of the *MYB-FL* mutation, pointing towards a lack of extensive pleiotropy. A similar specific phenotypic effect was observed for the mutation of the *ODO1* gene, demonstrated by the elimination of scent in the mutant (Chapter 4). Although *ODO1* expression differs from *MYB-FL* in that it correlates temporally with the fluctuation in emission of scent volatiles during 24 h (Verdonk et al., 2005), the knockdown phenotype is still fairly specific with only scent reduction observed. If genes targeted by selection control very specific spatial or temporal expression of downstream compounds in the pathways, they may be less likely to induce deleterious or disadvantageous pleiotropic effects when mutated, that could potentially be harmful to a plant's fitness. A mutation's relative contribution to adaptation will thus be negatively influenced if there are deleterious pleiotropic effects on fitness that arise (Wessinger and Rausher, 2012). By avoiding genes of extensive phenotypic pleiotropic effect and targeting genes with limited pleiotropic control and/or specific functions, such as R2R3-MYB transcription factors, mutations have a higher chance of conveying a positive fitness effect to plants and being fixed in a population.

A second and related hypothesis as to why R2R3-MYB transcription factors are preferentially targeted in speciation is not just their developmental placement, but their relative positions in transcriptional networks. If genes under selection are low in the regulatory hierarchy, the degree of pleiotropic effects occurring is reduced as their number of targets in the pathway is low. Both *MYB-FL* and *AN2* are the last known transcription factors in the hierarchy of the flavonoid biosynthetic pathway and have structural genes as targets. *MYB-FL* directly induces *FLS* (Chapter 3), which is one of the last enzymes needed for the production of stable flavonols (Albert et al., 2014; Esfeld et al., 2018). Its direct effect on one of the last genes in flavonol biosynthesis reduces the number of direct targets to a minimum. *AN2*, responsible for activation of the anthocyanin branch of the flavonoid biosynthetic pathway, is not quite as low in the regulatory hierarchy as *MYB-FL*, as more enzymes are still needed to achieve stable anthocyanins (Esfeld et al., 2018). However, it too is the last transcription factor of this branch of the pathway, as no further transcription factors act downstream of *AN2*. *ODO1* is higher in transcriptional hierarchy of the benzenoid-phenylpropanoid pathway than the two transcription factors involved in color synthesis (Verdonk et al., 2005; Amrad et al., 2016). It nonetheless regulates only several enzymes involved in scent volatile production and is not known to regulate genes important in the last steps of this pathway (Verdonk et al., 2005). Targeting such genes low in the regulatory hierarchy therefore seems to help eliminate unwanted pleiotropic effects.

An additional factor to explain the preferential targeting of R2R3-MYB transcription factors and elimination of pleiotropic effects may be genetic redundancy that can help buffer gene regulatory networks and make them highly robust (Nawy et al., 2005; Brady et al., 2011). Although the precise phenotypic changes caused by mutations in the transcription factors *MYB-FL* and *ODO1* do not suggest genetic redundancy, it may be important to avoid other pleiotropic effects that could arise. Perhaps the specific phenotypic effects we observed are due to a combination of specificity of the targeted transcription factors as well as genetic redundancy buffering other pleiotropic effects. Paralogues of R2R3-MYBs in color transitions have been detected in Solanaceae lineages as well as *Mimulus* (Cooley et al., 2011; Bliek et al., 2016) possibly contributing to this genetic redundancy. Our findings in the *myb-fl* mutant lines (Chapter 3) where the production of anthocyanins was activated in the petals of *Petunia* species with a non-

functional *AN2* gene may point towards some genetic redundancy being possible in the flavonoid biosynthetic pathway. For instance, a different transcription factor could be taking over the role of *AN2* to produce anthocyanins in the light pink colored *myb-fl* mutants, although known *AN2* orthologs (e.g. *DPL, AN4, PHZ*) do not show obvious elevated expression. Overall, a combination of transcriptional network position, pleiotropic effects, and redundancy may cause the preferential targeting of R2R3-MYB transcription factors during evolution.

Determining pollinator behavior by targeting single speciation genes

Current evidence for major shifts in pollinator behavior by targeting a single speciation gene in plants is sparse. Are shifts in pollination caused by major effect genes therefore improbable and more likely to be caused by mutations in many genes? Since evidence for genes of major effect influencing adaptation is accumulating (Doebley, 2004; Hoekstra et al., 2006; Nadeau et al., 2016; Todesco et al., 2020), I argue that it is more likely an issue of methodological constraints affecting the low number of studies being able to demonstrate major shifts in pollination due to speciation genes. Multiple plant systems along with *Petunia* have allowed for major strides in this field to be made, but the studies that have focused on genes of major phenotypic effect have a few major drawbacks: (1) Isogenic lines differing in only a single phenotypic characteristic caused by a mutation at a single locus are used for comparisons but the genes underlying these loci have not yet been determined to be speciation genes, (2) Introgression lines or near isogenic lines of plants are used where loci are narrowed down to include the region of interest but still include many other genes in the introgressed region with potential confounding effects, (3) Speciation genes have been determined but have not been tested at the level of reproductive isolation barriers (e.g. via pollinators) or (4) Natural variation in populations is found to be caused by genes of large effect but have not yet been analyzed between species. Our *Petunia* system allows us to address these methodological constraints in order to demonstrate reproductive isolation through pollinator behavior due to speciation genes.

Experiments with introgression lines have been widely used in multiple plant systems, allowing pollinator behavior based on differences due to an introgressed region to be

studied. Behavioral assays in *Mimulus* where alleles were substituted at the *YUP* flower color locus demonstrated differential pollinator preference but were done in near isogenic lines (Bradshaw and Schemske, 2003). Similarly, the impact of alterations in the *Rosea* and *Venosa* genes in *Antirrhinum* were also tested with pollinator behavioral assays using near isogenic lines (Shang et al., 2011), as well as the *AN2* locus in *Petunia* introgression lines (Dell'Olivo and Kuhlemeier, 2013). Even with generations of backcrossing, the genetic identity of these lines was still slightly lower than 100%. This may seem like a high percentage of genetic identity, but extrapolated to a plant's genome size or genomic regions defined by genetic linkage patterns, many genes still differ between the near isogenic lines being tested and the parental species (Dyer et al., 2007). For example, if a plant's genome consists of 20,000 genes, then a difference of 3% genetic identity after four generations of backcrossing would mean that 600 genes differ between the two species being tested, with only one of these genes being the target (Dyer et al., 2007). This is illustrated in Dell'Olivo and Kuhlemeier (2013), where the *Petunia* lines with targeted altered visible color also displayed morphological differences most likely due to nontargeted genes in the introgressed region. To be able to use these experiments to demonstrate reproductive isolation through a single and major effect gene, the causal gene(s) underlying the locus should be identified and functionally characterized. Using a precise molecular approach to target the speciation genes defined in our *Petunia* system allows us to overcome these potentially confounding factors for a more rigorous assessment of pollinator behavior based on differences in a single gene.

Different molecular approaches have been used to target genes and analyze pollinator behavior but have not yet succeeded in being able to do so for a single described speciation gene and both primary and secondary pollinators. The lack of light areas in *Mimulus* flowers have been shown to decrease visitation rates by bumblebees (Owen and Bradshaw, 2011). The inheritance pattern of the chemically induced mutation in this study was consistent with a single locus, but the causal gene was not identified (Owen and Bradshaw, 2011). The *LAR1* gene was later identified as a gene controlling spatial pattern variation of these regions between two sister species in the *Mimulus* genus (Yuan et al., 2016). Combining these two approaches to perform pollinator behavioral assays on *LAR1* mutant lines would allow the effect of this gene on reproductive isolation and its status as a possible speciation gene to be determined. A different gene in *Mimulus* has

been proposed to be a speciation gene: the floral scent gene *OS* (*OCIMENE SYNTHASE*). A reduction in *OS* expression through an RNAi approach led to reduced visitation by primary pollinators demonstrating a role of this gene in promoting reproductive isolation between two *Mimulus* species (Byers et al., 2014). To strengthen these observations, it would be interesting to perform behavioral experiments with the secondary pollinator (hummingbirds) to see how these phenotypic changes affects these pollinators. *Mixta* and *nivea* mutants in *Antirrhinum*, where single genes have been altered that affect flower color appearance, are one of the few examples where pollinator behavioral assays have been performed on single gene mutants, elegantly demonstrating their effects on pollinator behavior (Dyer et al., 2007; Whitney et al., 2009). However, these genes have not been defined as speciation genes in the *Antirrhinum* system as it has not been shown whether these genes confer differences between species and directly contribute to isolation between two species. Despite the difficulty in classifying a speciation gene as such and subsequently mutating it (in isolation) for pollinator behavioral assays, these studies offer extremely useful insights into the process of pollinator preference based on different phenotypic traits. In our *Petunia* system we are able to take these approaches one step further: In Chapter 3 I leveraged the CRISPR/Cas9 technique to mutate a single functionally characterized gene (*MYB-FL*) and determined its influence on reproductive isolation with both primary and secondary pollinators, thus meeting most criteria for speciation gene status. Being able to combine the findings using a single mutated speciation gene with further speciation genes (*ODO1, CNL1, AN2*), makes our approach in *Petunia* unique. *Petunia* is therefore truly a model system to analyze speciation events due to mutations of large phenotypic effect and precisely show how mutations in these genes affect pollinator behavior.

Towards a functional description of pollination syndrome transitions in *Petunia*

One major advantage of the mutations in various speciation genes in *Petunia* I have presented in this thesis, are their combinatorial possibilities. Although I have not yet tested all single mutations of speciation genes for their effect on primary and secondary pollinator behavior, it is to be assumed that these mutations would also elicit strong responses. For instance previous analyses using introgression lines with different scent

profiles (scented vs. non-scented) aimed to piece apart the volatile and visual stimuli that pollinators use to make choices (Klahre et al., 2011). This study showed that visual and olfactory cues seem to be equally important for attracting hawkmoth pollinators. However, open questions still remain. How does this affect a secondary pollinator? Are the individual contributions of UV and visible color also equal and how do these compare to olfactory stimuli? Using the system we have now set up would allow us to analyze these interactions in even closer detail, eliminating any possible confounding factors that were present in previous studies.

Specifically, we are now able to combine altered UV color, altered visible color and two different scent profiles through altered *ODO1* and *CNL1* genes in any possible combination. Pyramiding these traits and analyzing primary as well as secondary pollinator preference for every level would allow the magnitude of the individual traits on reproductive isolation to be quantified. For instance, we have already shown a major shift in primary pollinator preference for the *myb-fl* mutation (Chapter 3). This single mutation may be enough to deter primary pollinators but was not yet ideal for attracting secondary pollinators. If we can then show a major individual effect of either visual color or scent due to an altered single gene, this will help in verifying whether the proposed order of mutations due to phylogenetic studies are accurate.

Dissecting the importance of individual floral cues

As the individual effects of these genes could all cause major differences in pollinator preference, the analyses of combinations of these phenotypes in double and triple mutants will be highly important. For example if we observe a major shift in pollinator preference in all three genes *MYB-FL*, *AN2* and *ODO1*, we can ask ourselves whether this shift will be additive when combining the mutations or whether there will be a maximum difference in pollinator preference that can be reached? Even in nature, visitation by pollinators to *Petunia* species is not exclusive to a single pollinator. For instance, *P. axillaris* is mainly visited by nocturnal hawkmoths, but also receives bee visits during the day (Hoballah et al., 2007). If a major shift is observed with a single gene, every further mutated speciation gene would then help strengthen reproductive isolation but would no longer contribute anything additional towards this isolation barrier. Assuming this

proposed scenario, we would then need to observe if the double mutant combinations (*MYB-FL/AN2, MYB-FL/ODO1, ODO1/AN2*) all cause the same shift in pollinator preference. If they do, we could argue that all cues seem to be equally important for pollinator choice. However, if one of the combinations would lead to a weaker shift than the others, then we could conclude that the gene present in both of the other two combinations seems to elicit the strongest response. A gene conferring such a strong positive response to pollinators would probably be an ideal target as one of the first genes to have mutated during the shifts in pollination syndromes. On the other hand if there is one combination that elicits the highest response, then it would become clear that a combination of these genes is needed to lead to full pollinator isolation and that additive effects need to be considered.

Comparing these double mutants with the highest level of the pyramid, the triple mutants would allow these possible additive effects to be studied further. It is possible that a combination of two speciation genes is already enough to completely shift pollinator preference away from the primary pollinator towards the secondary pollinator and that further changes in speciation genes only help strengthen this shift. It is also plausible that certain phenotypic differences we find between *Petunia* species serve a dual effect: on the one hand they may be important for pollinator attraction but at the same time may be beneficial depending on other environmental factors. UV pigments help protect against abiotic stressors and can for example enhance pollen viability in regions of high UV-B radiation (Koski and Ashman, 2015a). Ultimately, combining these differences in pollinator behavior with the phylogenetic and molecular analyses that describe the shifts in pollination syndromes in *Petunia* thus far, would lead us towards a functional description of the evolutionary changes in the *Petunia* genus.

Evolutionary consequences of traits that act simultaneously

Analyzing the precise effect of combinations of these traits can also help determine whether certain traits can act as attracting traits for primary pollinators but may deter other pollinators guilds that can deplete a flower's resources without providing pollination services. In *Mimulus,* traits associated with hummingbird pollination were found to deter bee pollinators by imposing foraging costs on them (Gegear et al., 2017).

Traits with multiple simultaneous functions therefore have the potential to increase a plant's reproductive success. This may also be the case for traits that are linked and may therefore act together to attract a specific pollinator type. For example we observed a trade-off between flavonol and anthocyanin production in the flavonoid biosynthetic pathway. These traits can never be completely phenotypically decoupled due to the competition for substrates.

In the *myb-fl* mutant lines, mutating *myb-fl* resulted in UV reflective flowers, simultaneously freeing up precursors in the pathway to produce anthocyanins. Non-patterned purely UV absorbing flowers are usually important for nighttime pollinators, but do not have as strong of an effect on daytime pollinators (Chittka et al., 1994; White et al., 1994; Kelber et al., 2002; Chen et al., 2020). Through this loss-of-function mutation, an additional function is gained (visible color) to help attract a different pollinator group. This is supported by the behavioral assays (Chapter 3) where bees exhibited preference between the darker pigmented *P. axillaris* N *myb-fl* and wildtype lines but did not exhibit the same preference when asked to choose between the faintly colored *P. axillaris* P *myb-fl* mutant and wildtype lines (Fig. 3.9 and 3.10, Chapter 3). In this case, the *myb-fl* mutant loses its primary pollinator, the hawkmoths, while gaining access to a new pollinator class (bees preferred *myb-fl* mutants over *P. axillaris* wildtype lines). Combining the findings for the flavonol/anthocyanin trade-off with the behavioral assays allows inferences to be drawn about the order of events that happened during the speciation process: *MYB-FL* was most likely the first color gene to have mutated during the hawkmoth to bee transition in the long tube clade. This loss-of-function mutation was likely a prerequisite for the success of subsequent mutation in *AN2* to have any effect on anthocyanin levels and subsequent pollinator visitation.

Subtle UV variation as a component affecting pollinator attraction

A further important aspect to consider regarding pollinator attraction via UV color is how UV color is quantified. We have adopted a binary approach, where we either classify a flower to be UV absorbent or UV reflective, as is the case for most *Petunia* species (e.g. *P. axillaris* and *P. secreta* respectively). However, there are many cases of UV patterning in nature that affect pollinator attraction (Horth et al., 2014; Koski and Ashman, 2014,

2015a, 2015b) and *P. inflata* in the short tube clade also displays a UV bullseye pattern (Esfeld et al., 2018; Fig. 2B). A UV bullseye pattern is defined as the petal bases being UV absorbent while the petal tips are UV reflective. Such UV patterns have been shown to increase diurnal pollinator attraction (Johnson and Andersson, 2002; Koski and Ashman, 2014). The extent of UV absorbing to UV reflecting area in this pattern may vary and these differences can also influence pollinator visitation (Horth et al., 2014; Koski and Ashman, 2015a, 2015b). The loss of UV patterning in the long tube clade may therefore be a further factor influencing pollinator shifts in *Petunia*.

If pollinators are able to perceive subtle variations in UV bullseye patterns, they may also be able to perceive variation in UV absorbance (and therefore flavonol concentration) in different *Petunia* species. Although we did not detect any significant differences in flavonol absorbance of *P. axillaris* N and P *myb-fl* mutants, there was substantial variation in quercetin levels of tested *P. axillaris* N samples (Fig. 3.3 A, Chapter 3). Also, comparing UV photographs of the *myb-fl* mutants from both *P. axillaris* lines (Fig. 3.2 B, Chapter 3) points towards possible differences in intensity and thereby pollinator perception of UV color. This would also be in line with the flavonol/anthocyanin trade-off in the biosynthetic pathway, where a higher level of residual flavonols in the *P. axillaris* P mutant would allow a lower amount of anthocyanins to be produced through competition for precursors. If we are able to detect subtle differences in the UV photographs by human eye, then pollinators who can perceive UV ranges in the color spectrum will most likely detect differences as well. This may point towards a more detailed UV color classification system being needed to further identify subtle UV variations possibly influencing pollination behavior.

Different pollinator guilds may exert different selective pressures

The work presented in this thesis will be important for carrying out further pollinator behavioral assays, ideally under natural conditions with native pollinators once precise greenhouse experiments have been conducted. Behavioral assays with both bumblebees and solitary bees demonstrated differences in foraging strategies of the different bee species. Such controlled greenhouse environments are ideal to determine the effect on pollinator preference of the individual phenotypic traits in all plant lines but lack the

ability to determine how native pollinators in natural environments will react to different plants. Visitations to *P. secreta* are scarce (Rodrigues et al., 2018) and bees of the genus *Pseudogapostemon* have not been widely studied. Foraging strategies of different bee species may greatly affect a plant's reproductive success and these different foraging strategies may be associated with differences in color learning (Muth et al., 2015; Nicholls et al., 2015). When provided with either sucrose or pollen as a reward, differences in learning mechanisms were found for the tested bees (Nicholls et al., 2015). Bees are also able to rapidly learn to discriminate between different colors and associated colors with either a nectar or pollen reward offered (Muth et al., 2015). Such studies demonstrate the complex learning behavior that can be associated with pollinator preference and that pollinator choice should never be viewed as a black/white decision. Testing our developed plant lines in the field would therefore allow further data to be collected on the ecological impact of these gene mutations and the preference of the native pollinator species.

Concluding remarks

In this thesis I aimed to mutate *MYB-FL* in *Petunia*, to specifically alter the UV color phenotype and combine this with further mutated major effect genes to mimic the transition from hawkmoth to bee pollination. Through pollinator assays I was able to demonstrate that mutations to a single gene (*MYB-FL*) are sufficient to cause a major shift in pollinator preference. Expanding these findings to further mutated speciation genes, should strengthen the shift in pollinator preference observed, leading to reproductive isolation through pollinator preference. Demonstrating the steps that occurred during *Petunia* speciation is not possible in real time, but the speciation gene system in *Petunia* offers a unique opportunity to recreate evolution in a laboratory setting. Ultimately, combining all of the identified speciation genes to create a true *P. secreta* mimic that we could test with natural pollinators would allow us to settle the longstanding debate on whether speciation can occur through a few genes of major effect.

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Declaration of consent

On the basis of Article 18 of the PromR Phil.-nat. 19

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Study program: PhD in Molecular Life Sciences

Bachelor ☐ Master ☐ Dissertation ■

Title of the thesis: Reconstitution of pollinator-mediated speciation in *Petunia* through single gene mutations

Supervisor: Prof. Dr. Cris Kuhlemeier

I declare herewith that this thesis is my own work and that I have not used any sources other than those stated. I have indicated the adoption of quotations as well as thoughts taken from other authors as such in the thesis. I am aware that the Senate pursuant to Article 36 paragraph 1 litera r of the University Act of September 5th, 1996 and Article 69 of the University Statute of June 7th, 2011 is authorized to revoke the doctoral degree awarded on the basis of this thesis.

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